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# THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

## X. AN EXPERIMENTAL TEST OF SOME ASPECTS OF THE TEORELL AND MEYER-SIEVERS THEORIES OF ELECTRICAL MEMBRANE BEHAVIOR

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(Received for publication, April 21, 1944)

### I

In several recent publications<sup>1-6</sup> we have shown experimentally that the acidic groups present in collodion are the primary cause of its electrochemical activity in solutions of strong inorganic electrolytes. The surface concentration of these acidic dissociable groups is assumed to determine the degree of electrochemical activity of any given collodion sample. The abundance of these groups varies with the past history of the collodion from which the membranes are prepared.

This concept of the cause of the electrochemical membrane activity is in general agreement with some newer theories of electrochemical membrane activity, outlined independently by Teorell<sup>7</sup> and by Meyer and Sievers.<sup>8</sup> On the basis of these theories it can be concluded that the base exchange capacity of the pore surfaces of a given membrane and its porosity are the factors determining its electrochemical activity. The theory also allows quantitative predictions by which one can link in a definite way base exchange capacity and electromotive behavior.

The empirical correlation between the base exchange capacity of various collodion preparations and their electrochemical activity has been described previously<sup>4</sup> in connection with studies of the base exchange properties of precipitated fibrous collodion. Such preparations have a very large specific surface and are suitable objects from which to obtain basic information. Our experimental results can be summarized by stating that there is no necessary

<sup>1</sup> Sollner, K., and Abrams, I., *J. Gen. Physiol.*, 1940, **24**, 1.

<sup>2</sup> Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **24**, 467.

<sup>3</sup> Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **25**, 7.

<sup>4</sup> Sollner, K., Carr, C. W., and Abrams, I., *J. Gen. Physiol.*, 1942, **25**, 411.

<sup>5</sup> Sollner, K., and Carr, C. W., *J. Gen. Physiol.*, 1942, **26**, 17.

<sup>6</sup> Sollner, K., and Carr, C. W., *J. Gen. Physiol.*, 1943, **26**, 309.

<sup>7</sup> Teorell, T., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 282; *Proc. Nat. Acad. Sc.*, 1935, **21**, 152.

<sup>8</sup> Meyer, K. H., and Sievers, J.-F., *Helv. Chim. Acta*, 1936, **19**, 649, 665, 963.



correlation between base exchange capacity of collodion and its electrochemical activity. There are preparations which combine (a) immeasurably low base exchange capacity and low electrochemical activity, (b) immeasurably low base exchange capacity and high electrochemical activity, (c) measurable base exchange capacity and high electrochemical activity, (the activity in this case is not necessarily higher than in the preceding one).

In later experiments Sollner and Anderman<sup>9</sup> have made a study of the total acidity, the "acid number," of similar preparations. A qualitative parallelism between the electrochemical activity and total acidity was found; it also was shown that the total acidity did not vary in the same manner as the base exchange capacity of the different collodion samples.

These results were contrary to the original expectation, and, more important, they seemed incompatible with the Teorell<sup>7</sup> and Meyer-Sievers<sup>8</sup> theories. The present investigation is an attempt to clarify this situation on a quantitative basis. The base exchange capacity of membranes is compared with what should be an identical or at least a similar value calculated according to the Teorell and Meyer-Sievers theories on the basis of potentiometric measurements.

A necessary condition for the applicability of these theories to membranes of a porous character is their rigidity; *i.e.*, their inability to swell appreciably under the experimental conditions. We have shown previously<sup>10</sup> that both porous and water-wetted dried collodion membranes can be considered rigid structures in solutions of strong inorganic electrolytes.<sup>11</sup> They can therefore be used to test the theory.

Before the experiments are described, a short exposition of the Teorell and Meyer-Sievers theories must be given. In substance the two concepts are identical. Teorell has given only brief accounts of his theory; Meyer and Sievers go much more into detail and have tried to corroborate their ideas by experiment. This theory is today the outstanding example of an attempt to put on a rational basis perhaps the most important electrochemical function of membranes, their electromotive action.

Since the Teorell, Meyer-Sievers theory is of a highly involved nature, it is preferable to give a short outline of it as presented by Meyer.<sup>12</sup>

"Consider a membrane consisting of an acid high-molecular substance, for instance of pectin chains, of which the carboxyl groups have been neutralised with metallic

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<sup>9</sup> Sollner, K., and Anderman, J., *J. Gen. Physiol.*, 1944, **27**, 433.

<sup>10</sup> Carr, C. W., and Sollner, K., *J. Gen. Physiol.*, 1943, **27**, 77.

<sup>11</sup> Air-dried collodion membranes swell to a slight extent when placed in water, but do not change in structure when transferred from water to solutions of strong electrolytes. Therefore, for the present purpose they can be considered as rigid non-swelling structures.

<sup>12</sup> Meyer, K. H., *Tr. Faraday. Soc.*, 1937, **33**, 1073.

cations, *e.g.*, potassium ions. The membrane then possesses fixed anions and mobile cations. The cations may therefore be displaced if a supply of others is maintained from one side: the membrane is cation permeable. The concentration of the fixed anions, calculated in gram equivalents per litre of the imbibed liquid, is a quantity characteristic for each membrane which we will call the "selectivity constant,"  $A$ . If now the membrane be immersed in a salt solution, both ions of the salt will penetrate into it; the equilibria then obtaining may be calculated from the Donnan equation: the actual membrane behaves like a solution bounded by two ideal Donnan membranes through which the fixed ions cannot pass. . . ."

"If a current is passed across the membrane, the transport of the electricity will be divided between the two kinds of mobile ions in accordance with the relative numbers of ions passing through the membrane. The ratio,  $n_c/n_A$  between the numbers of cations and of anions traversing the membrane, which we will call the ratio of the transport or "traversal" numbers, may be determined by the same methods as those used for the determination of transport numbers in a solution.  $n_c/n_A$  depends on the rates of migration of the mobile ions and on their number; as mentioned above, the latter is dependent on the concentration of the ions in the surrounding liquid. We then obtain

$$\frac{n_c}{n_A} = \frac{U_c \cdot (y + A)}{U_A \cdot y} = \frac{U_c}{U_A} \cdot \frac{\sqrt{4c^2 + A^2} + A}{\sqrt{4c^2 + A^2} - A} = \frac{U_c}{U_A} \cdot R,$$

where  $U_c$  and  $U_A$  are the rates of migration,  $c$  the molar concentration of the salt in the surrounding liquid, and  $A$  the selectivity constant. . . .  $y$  is the concentration of mobile anions in the membrane.

"The dependence of the selectivity, *i.e.* of the quantity  $n_c/n_A$ , on the concentration is expressed by the factor  $R$ . Its dependence on the ratio  $c/A$  is shown by the following table:

$c/A$	10	1	0.1	0.01	0.001
$R$	1.1	2.6	101	10,000	1,000,000

"If we therefore take a membrane with wide pores such that the concentration of the fixed ions in its aqueous parts is normal ( $A = 1, c^2/A = c^2$ ) and surround it with a salt solution the ions of which have equal mobilities, then  $R$  will equal the selectivity  $n_c/n_A$ . The dependence of  $n_c/n_A$  on the external concentration has, as mentioned above, been long known (the "concentration effect"), but so far without having been explained.

"Ionic selectivity is not, however, the sole factor governing ionic permeability: in a network the "sieve effect" can also occur, its importance being the greater the finer the mesh of the net; this will in general be the case with membranes which contain little liquid of imbibition. Finally the "solubility" of the ions in the membrane may also play a part. By this we mean that, as a result of the attractive influence of the organic groups of the membrane, some, say organic, ions may attain a greater concentration in the aqueous liquid of imbibition of an organic membrane than in pure water. The complete equation therefore reads

$$\frac{n_c}{n_A} = \frac{U_c}{U_A} \cdot \frac{\sqrt{4c^2 l_c l_A + A^2} + A}{\sqrt{4c^2 l_c l_A + A^2} - A},$$

where  $U_C/U_A$  is the ratio of the rates of migration in the membrane under the influence of the sieve effect, and  $l_C$  and  $l_A$  the solubility coefficients (partition coefficients) of the ions with respect to membrane and water.

"Now these two essential properties of the membrane—its sieve action with respect to different ions (as expressed by the quotient  $U_C/U_A$ ) and its selectivity constant, can be determined by measuring the traversal numbers at different concentrations. The potentiometric method is the best; the potential set up when the membrane separates two solutions of the same salt, but of different concentrations is measured, the absolute concentrations being varied in such a way that their ratio is kept constant. When there is no ionic selectivity the potential is determined only by the quantity  $U_C/U_A$ , which is dependent on the absolute concentration; the greater the value of  $A$  as compared with the external concentration, the more marked will be the ionic selectivity.

" $A$ , the selectivity constant, and  $U_C/U_A$ , which includes the expression for the sieve effect, can be quantitatively determined either by calculation or graphically. We will not here reproduce the complicated formulae required for the calculation, but will merely give a short account of the graphical method.

"Curves are constructed plotting as ordinates the potential differences measured between two solutions of the same binary electrolyte, the concentrations ( $c_1, c_2$ ) of which are always as 1:2, and as abscissae the quantity  $\log \frac{A}{c_1}$ . For an electrolyte, the two

ions of which have equal mobilities in the membrane (*i.e.*,  $U_C/U_A = 1$ ), a certain curve will then be obtained while other values of  $U_C/U_A$  will result in other curves. . . ." (The calculated curve for  $U_C/U_A = 1$  is given below in Figs. 1 and 2.)

"To determine  $A$  and  $U_C/U_A$  for an unknown membrane it will then only be necessary to determine several values of  $E$  for different absolute values of  $c_1$ , the concentration  $c_2$  being always kept equal to  $2c_1$ . The observed values of  $E$  are then plotted against  $c_1$  (ordinates) using the same coordinates as before, and then the experimental curve is displaced sideways (parallel to the abscissa) until it has been successfully brought into coincidence with one of the curves already drawn; interpolation may be necessary in this procedure. In this way  $U_C/U_A$  is determined, the value depending only on the shape of the curve. The amount of the displacement as read off on the abscissa gives  $\log A$ , and therefore  $A$ . . . ."

This quantity, " $A$ ," according to Meyer and Sievers can be determined on the basis of potentiometric measurements. Values thus obtained will be designated as  $A_p$  throughout this paper.

On the other hand, " $A$ " by definition is the concentration of the fixed ions in the aqueous part (pore space) of the membrane, or more correctly the concentration calculated from the number of equivalents of anions fixed immovably to the pore walls, divided by the pore space (in liters) of the same membrane.

Electrically these anions are compensated for by an equivalent quantity of cations. These cations, potassium ions in the example given by Meyer, can be replaced by other cations if the membrane is brought into a suitable electrolyte solution. This actually is the mechanism which according to the theory makes the membrane behave in its characteristic manner with different cations.

In other words, the membrane must show the phenomenon of base exchange in order to exhibit its electromotive properties.

If one is able to determine the base exchange capacity of the membrane, and if one knows its pore space, one is able to calculate the " $A$ " value of the Meyer and Sievers theory,<sup>13</sup> " $A$ " being the base exchange capacity in equivalents divided by the pore volume in liters. The " $A$ " values which are calculated from base exchange studies will be denoted  $A_b$ .

If the theoretical assumptions on which the theory is based are correct,  $A_p$ , derived from potential measurements must be identical with  $A_b$ , derived from base exchange studies. A comparison of the " $A$ " values obtainable in these two independent ways is the subject of the present investigation.

Before approaching this subject, however, a few critical remarks should be made concerning the Teorell, Meyer-Sievers theory.

Excellent agreement with the experimental facts has been claimed for this theory in several respects, particularly the independence of the " $A$ " value ( $A_p$ ) of concentration. Actually the constancy of  $A_p$  is as good as claimed only in rare cases, as was shown by Fetcher<sup>14</sup> for Meyer and Sievers own experimental data.

The constancy of  $A_p$  and its independence of the nature of the electrolyte is bound to so many conditions that it can hardly be expected to hold true. Firstly, it assumes a degree of ionization of the fixed ionizable surface compounds independent of the nature of the counter-ions and of the concentration of the electrolyte solution. This can hardly be the case even when comparing monovalent cations; the hydrogen ion certainly should behave much differently for the dissociation of the acidic surface compounds ("nitrocellulosic" acid with collodion) undoubtedly is much less than that of the corresponding alkali salts.

With membranes having only small pores an additional difficulty arises. The electrolyte content of dried collodion membranes after several days' contact with solutions of different chlorides varies with the cation. With hydrochloric acid and potassium chloride, the electrolyte content of the membranes was found to be about twice as great as with lithium chloride.<sup>15</sup> Presumably

<sup>13</sup> This procedure involves the assumption that the number of exchangeable, monovalent cations is identical with the number of dissociated, immovable electronegative groups; in other words it assumes complete ion exchange and complete dissociation of the dissociable surface compounds. In view of the completeness of the ion exchange and of the high degree of dissociation of the alkali salts of even weak organic acids these assumptions appear to be permissible. It will be seen below that any error which possibly could be introduced in this manner is too small to affect to a significant extent any conclusions drawn from the experimental results.

<sup>14</sup> Fetcher, E. S., Jr., *J. Physic. Chem.*, 1942, **46**, 570.

<sup>15</sup> Green, A. A., Weech, A. A., and Michaelis, L., *J. Gen. Physiol.*, 1929, **12**, 473.

these membranes contain pores inaccessible for the lithium ion, but accessible for potassium, hydrogen, and chloride ions. There seems to be little doubt that only a part of the pores which are electromotively active with hydrochloric acid and potassium chloride, are electromotively active with lithium chloride. Identity of the  $A_p$  values with the various electrolytes would, therefore, be fortuitous, at least from the point of view of the theory.

It must be recognized that at least some of these difficulties could be overcome by proper corrections and additions to the Teorell, Meyer-Sievers theory, which would result in a better agreement between the theoretical and experimental curves.

The main question of general interest which arises is whether the Teorell, Meyer-Sievers theory is inherently a correct representation of the physical facts which lie behind the observable potential-concentration relationships, or whether it is only a formal way of bringing these relations into a fictitious, formally correct framework. It is clear that the planned test of the agreement between the selectivity constants  $A_p$  and  $A_b$ , as established by the two entirely different methods would materially help to decide this question in one or the other way.

## II

According to the above outlined plan two kinds of measurements were performed with the same membranes. First the potentiometric studies were made; then the base exchange was determined, and in order to obtain the water content, the wet and dry weights of the membranes were measured. From these data the selectivity constants,  $A_p$  and  $A_b$ , were obtained as explained in principle above.

In order to cover a wide range of membrane porosities and activities the following kinds of membranes were investigated: (a) membranes prepared from collodion preparations of different activity; (b) "oxidized membranes."<sup>3</sup>

In the case of the first of these groups "dried" and "porous" bag-shaped membranes were prepared from the collodion preparations characterized in Table I which gives the brand and pre-treatment (if any) of the various preparations together with the data on their electrochemical activity. The membranes were cast in  $30 \times 110$  mm. test tubes from 5 per cent collodion solutions in absolute ether-alcohol (75:25); the porous membranes were immersed in water after drying for only 20 minutes, the "dried" ones after 24 hours drying time.

The second group of membranes, the "oxidized membranes" were prepared in the following manner. "Dried" membranes cast from commercial collodion were swelled in alcohol of varying (85, 90, and 95 per cent) concentration. The swelled membranes were washed with water, oxidized with 1 M NaOBr for 3 hours, and rewashed thoroughly. In this way a series of oxidized membranes was obtained with water contents of 17, 24, and 62 volume per cent. In all cases at least three membranes of each kind were prepared.

For the *potential measurements* one membrane only of each kind was used since it was shown in preliminary experiments that the variations within each set of identically prepared membranes were negligible. The potentials were

TABLE I  
*Some Characteristics of Various Collodion Preparations\**

1	2	3	4	5	6	7	8	
	Brand of collodion and pretreatment  (All preparations were precipitated from ether-alcohol solutions and dried)	Base exchange data (48 hrs.)		Acid number 0.01 N KOH per gm. dry collodion (corrected for ash content)	Electrochemical activity			
		0.01N NaOH per gm. dry collodion on treatment with 0.5 M KCl	pH values on treat- ment with 0.5 M KCl		Anomalous osmosis (porous membranes†)	Osmotic rise with 0.25 M sucrose	Anomalous osmotic rise with $\frac{M}{512}$ K <sub>2</sub> SO <sub>4</sub>	Character- istic con- centration potential 0.1 M KCl/ 0.01 M KCl (dried mem- branes‡)
					ml.	ml.	mm.	mm.
1	Mallinckrodt "Par- lodon," boiled 15 hrs. in 90 per cent alcohol	0.01	5.9	1.0	106 128 136	15 16 28	27 28 28	
2	Baker Collodion U.S.P., commercial preparation	0.03	5.3	1.5	126 128 130	50 32 47	41 42 42	
3	Oxidized collodion; Baker collodion cotton, "Pyrox- ilin" oxidized 48 hrs. with 1 M NaOBr and boiled several times with water	0.27	4.2	3.3	118 130 137	130 142 148	51 52 53	
4	Oxidized collodion (No. 3), washed 16 times with 95 per cent alcohol	0.03	5.0	1.6	132 137 140	119 128 131	49 50 52	

\* The base exchange experiments summarized in Table I were performed with precipitated fibrous collodion as in previous work.<sup>4</sup> The acid numbers were obtained using the method previously described by Sollner and Anderman.<sup>9</sup> The anomalous osmosis and concentration potentials were determined as described in previous papers.<sup>1-4</sup>

† The porous membranes described in columns 6 and 7 are of course not the same specimens as the dried ones characterized in column 8.

measured with potassium chloride solution of one concentration inside the membrane and a potassium chloride solution of another concentration outside. The concentration ratio was always 2:1 while the absolute concentrations varied between 0.64/0.32 to 0.0050/0.0025 M. In the case of the "porous" membranes, the solutions were stirred vigorously by bubbling air through them

to minimize the effects due to diffusion of electrolyte or movement of solution in bulk (essentially by anomalous osmosis) from the more concentrated solution into the less concentrated one. The solutions were also reversed in a few cases to make sure that the membranes were symmetrical; this was true for all those tested. The results of these potential measurements are plotted in Figs. 1 and 2. Also included in the figures is the curve of Meyer and Sievers calculated for KCl ( $U/V = 1$ ) and  $A_p = 1$ . As indicated above this is the standard

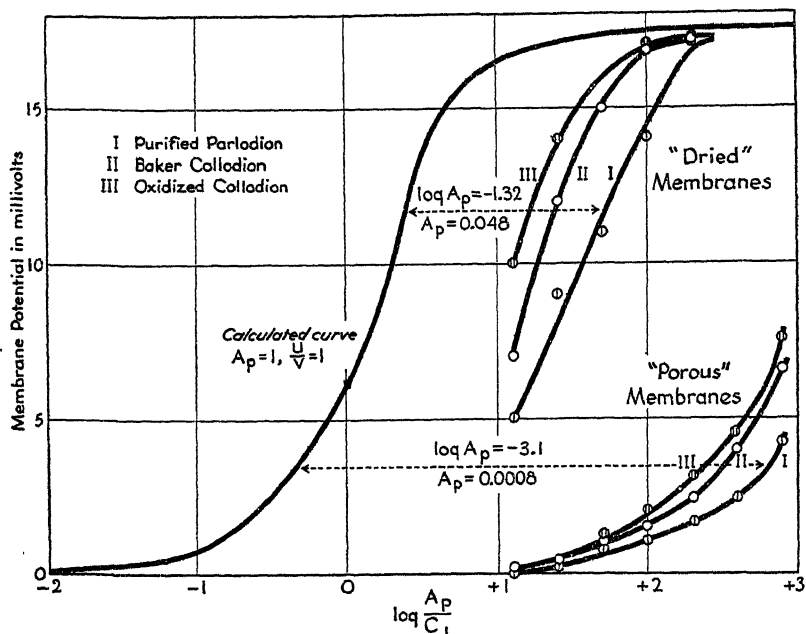


FIG. 1. Potential /  $\log \frac{A_p}{C_1}$  curves of "dried" and "porous" membranes prepared from various collodion preparations.

curve, by reference to which  $A_p$  for any membrane is determined. The upper three experimental curves in Fig. 1 are for the dried membranes while the lower three are for the porous membranes. Curves for the purified oxidized collodion (No. 4 in Table I) are not shown because they almost coincide with the curves for the unoxidized (Baker) collodion. Fig. 2 shows the results obtained with "oxidized membranes" of varying porosity.

The logarithms of the  $A_p$  values can be read directly from the graphs.  $\log A_p$  is the horizontal distance between the standard curve and the experimental curve. If these curves are on the right side of the standard curve, this value is negative. Since even the steeply ascending branches of the experimental

curves are not strictly parallel with the theoretical curve a mean  $A_p$  value for each curve must be chosen. These  $A_p$  values for dried and porous membranes cast from the various collodion preparations (characterized in Table I) are listed in column 8 of Table II, and for "oxidized membranes" in Table III. As will be seen below the uncertainty of the  $A_p$  values, which is due to the shape of the experimental curves, is of no consequence for the conclusions which are based upon them.

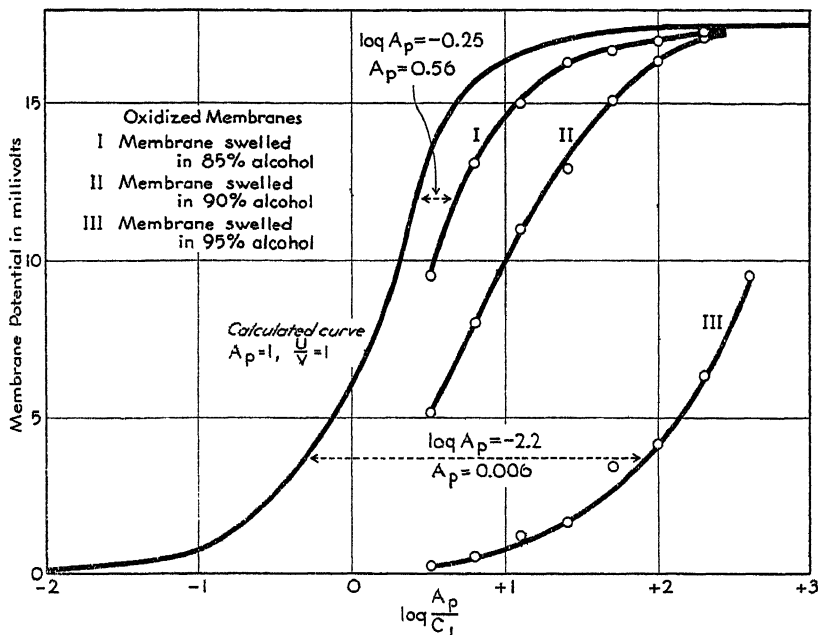


FIG. 2. Potential /  $\log \frac{A_p}{C_1}$  curves of "oxidized membranes" of various porosities.

After the potential measurements were completed, the *base exchange capacity of the membranes* was determined. The general principles of the base exchange method and its application to collodion have been discussed in a previous paper<sup>4</sup> and need no further comment here. The only change in the procedure was that in the present experiments the base exchange capacity of *membranes* was measured, not the base exchange of fibrous collodion as was done previously.

The base exchange experiments were carried out with three membranes of each kind in order to have sufficiently large samples. The membranes that had been used for the potential measurements were first returned to the acidic state as described for fibrous collodion in previous papers.<sup>4, 5</sup> For the base exchange the membranes were immersed in 25 ml. of 0.5 M KCl solution.



After 24 hours contact with the membranes the pH of the solution was determined with a glass electrode. Control experiments were also run with water in place of the electrolyte solution. The pH values of the KCl solution and of the water are reported in columns 5 and 6 of Tables II and III.

For many of the membranes tested, the change in pH was very small and moreover the same for the water as for the KCl solutions.<sup>16</sup> Nevertheless, in these cases the pH data in column 5 were used in calculating the  $A_b$  values.

Finally the *water content* of these membranes was determined. The wet membranes were removed from their glass rings, cut open, blotted dry of surface water, and weighed in a closed weighing bottle. Then they were dried over sulfuric acid and reweighed. The weight loss represents the water content.<sup>17</sup> The water content of the membranes in volume per cent is given in column 3 of Tables II and III. The absolute water content of the various experimental samples is given in column 4.

The calculation of  $A_b$ , the base exchange selectivity constant, is based on the following consideration.  $A_b$  by definition is equal to the concentration of the fixed ionizable groups in the water contained in the membrane, which is equal to the concentration of the counter-ions of the fixed groups. The counter-ions,  $H^+$  ions in our case, are determined experimentally after exchange with the  $K^+$  ions of a known volume of KCl solution; being contained now in the KCl solution their concentration in the latter is measured electrometrically. The concentration of the counter-ions of the fixed dissociable groups (and therefore the concentration of the latter) in the water contained in the membrane can therefore be calculated from the following equation.

$$C_m \times V_m = C_s \times (V_s + V_m)$$

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<sup>16</sup> For detailed discussions of this point see: Sollner, K., Carr, C. W., and Abrams, I., *J. Gen. Physiol.*, 1942, **25**, 411; and Sollner, K., and Anderman, J., *J. Gen. Physiol.*, 1944, **27**, 433.

<sup>17</sup> The water content of collodion membranes represents only a maximum value for the available pore space. Some water, undoubtedly, is "bound" to the collodion and not available for the typical membrane functions. With "porous" membranes of high water content the fraction of "bound" water is negligibly small; with dried membranes the "bound" water may be a sizable fraction of the total water content (Carr, W. C., and Sollner, K., *J. Gen. Physiol.*, 1943, **27**, 77). In the calculations reported below which involve the pore water content of the membranes the assumption is made that the total water content is pore water. If we make the exaggerated assumption that 50 per cent of the water content of the "dried" membranes is "bound" water the figures for cases 1 to 4 in column 7 of Table II would be twice as large as given, and the figures in column 9 would be half of the present values. This, however, would in no way affect the essence of the conclusions which will be drawn below from these figures.

$C_m$  = Concentration of fixed ionizable groups in the water contained in the membrane.

$V_m$  = Volume of  $H_2O$  in the membrane (column 4 of Tables II and III).

$C_s$  = Concentration of HCl in the KCl solution (see column 5 of Tables II and III).

$V_s$  = Volume of the KCl solution (25 ml.).

Since by definition  $C_m = A_b$ ,

$$A_b = \frac{C_s \times (V_s + V_m)}{V_m}; \quad (2)$$

as  $V_m \ll V_s$  we may use the simplified equation

$$A_b = \frac{C_s \times V_s}{V_m} \quad (3)$$

The base exchange selectivity constants  $A_b$  so calculated are recorded in column 7 of Tables II and III.

The fact that many of these  $A_b$  values are only maximum values is indicated by a  $<$  sign.

Column 9 of Tables II and III gives the end result of the foregoing experiments and calculations, namely the comparison between the potentiometric selectivity constant  $A_p$  and the base exchange selectivity constant  $A_b$ ; it is given as the ratio  $\frac{A_p}{A_b}$ .

### III

We turn now to the main objective of this paper, namely the comparison of the  $A_p$  and  $A_b$  values, the ratio of which is given in column 9 of Tables II and III for eleven different cases.

In prior sections of this paper it was pointed out that the Teorell, Meyer-Sievers theory leads to the anticipation of an identity of the selectivity constants, as determined by the potentiometric and base exchange methods. The ratio  $\frac{A_p}{A_b}$  should be one, or, considering the uncertainties of the theory, this ratio should not deviate too strongly from unity.

The first glance at the  $\frac{A_p}{A_b}$  ratio in the last column of Tables II and III, however, shows that this is by no means the case. The values of the  $\frac{A_p}{A_b}$  ratio are spread over three orders of magnitude, varying from 0.08 to 107.

In view of the fact that the agreement between the  $A_p$  and  $A_b$  values is much better with the "oxidized membranes" (Table III) than with the membranes prepared from various collodion preparations (Table II) we shall treat the two groups separately.

TABLE II  
*A<sub>b</sub> and A<sub>p</sub> Values of Membranes Prepared from Various Collodion Preparations*

1	2	3	4	5	6	7	8	9
	Brand of collodion (see Table I) and type of membrane	Water content of mem- branes	Base exchange data			A <sub>b</sub> calculated from base exchange data (see columns 4, 5, and 6)	A <sub>p</sub> obtained from exper- imental potential curves of Fig. 1	$\frac{A_p}{A_b}$
		vol. per cent	gm.			equiv- alents/l. × 10 <sup>3</sup>	equiv- alents/l. × 10 <sup>3</sup>	
1	Purified "Par- lodian," dried membranes	8.2*	0.029	5.8	5.9	<1.4*	48	>34*
2	Baker Collodion U.S.P., dried membranes	10.5*	0.022	5.9	5.8	<1.5*	100	>67*
3	Oxidized collodion, dried membranes	10.5*	0.023	5.9	5.9	<1.4*	150	>107*
4	Oxidized collodion, purified, dried membranes	10.5*	0.021	5.9	6.0	<1.6*	100	>62*
5	Purified "Par- lodian," porous membranes	80	0.821	5.8	5.8	<0.05	0.8	>16
6	Baker Collodion U.S.P., porous membranes	75	0.626	6.2	6.0	<0.02	1.5	>75
7	Oxidized collodion, porous mem- branes	79	0.339	5.4	6.0	0.3	2.0	6.7
8	Oxidized collodion, purified, porous membranes	77	0.556	6.0	6.2	<0.045	1.5	>33

\* Compare footnote 17.

TABLE III  
*A<sub>b</sub> and A<sub>p</sub> Values of Various "Oxidized Membranes"*

1	2	3	4	5	6	7	8	9
	Concentra- tion of alcohol used for swelling of membranes	Water content of membranes	Base exchange data			A <sub>b</sub> calculated from base exchange data (see columns 4, 5, and 6)	A <sub>p</sub> obtained from exper- imental potential curves of Fig. 2	$\frac{A_p}{A_b}$
	per cent	vol. per cent	gm.			equiv- alents/l. × 10 <sup>3</sup>	equiv- alents/l. × 10 <sup>3</sup>	
1	85	17	0.030	3.4	6.0	330	560	1.8
2	90	24	0.045	3.4	5.9	220	170	0.77
3	95	62	0.203	3.2	6.1	78	6	0.08

With the membranes listed in Table II the given  $A_b$  values are smaller by one to two orders of magnitude than the  $A_p$  values. The discrepancy between the results for the two different methods is in reality greater because the calculation of  $A_b$  is based upon experimental data which represent the upper limit of the exchange capacity of the membranes. These values—except with case 7—are, as was explained before, fictitiously high. It is impossible at present to estimate with any degree of accuracy the influence of this factor, but the true base exchange capacity—except in case 7—may be smaller by orders of magnitude; the discrepancy between the two  $A$  values would be increased correspondingly.

There is also another factor which tends to increase the discrepancy between  $A_p$  and  $A_b$ . Sollner and Anderman<sup>9</sup> have shown that the time required for reaching the maximum base exchange even with fibrous collodion is 24 hours, the base exchange after 30 minutes being only about 5 to 20 per cent of the final value. Evidently a large number of the active groups lie in the interior of the micelles or in dead-end cavities in which the exchange can take place only very slowly. On the other hand, if membrane potentials are measured the final potential values are established after a few minutes of contact with the electrolyte solution. The number of active groups responsible for the potentials, therefore, must be smaller probably by at least one order of magnitude than the number determined by the base exchange after 24 hours upon which our calculations are based. If this time effect is considered, as it must be, the ratio " $A_p$ "/" $A_b$ " becomes larger by at least one order of magnitude than the values given in column 9 of Table II, quite apart from the influence of the above mentioned other factor. The true discrepancy between the  $A_p$  and  $A_b$  values therefore is not one to two orders of magnitude as indicated in the last column of Table II,  $A_b$  is in reality at least two to three orders of magnitude smaller than  $A_p$ . If the first of the two factors which tend to increase this discrepancy could be evaluated quantitatively, the disagreement would be found to be still greater.

The results with the "oxidized membranes" in Table III indicate a better agreement between the two methods of determining the selectivity constant than that obtained with the first group of experiments. In one case (No. 3 of Table III)  $A_b$  is larger than  $A_p$  by a factor of 13.

It must be emphasized that contrary to the situation with the preceding group of membranes, the base exchange values with the "oxidized membranes" are believed to be correct base exchange capacity values, not falsified by any known error. The first factor which in the former cases tended to decrease the  $A_b$  values is therefore here not operative. However, the time effect on the base exchange must be considered; if this is done  $A_b$  values become much lower. Thus for the one case (No. 3) the agreement between  $A_p$  and the corrected  $A_b$  value may be satisfactory, but with the other two cases (Nos. 1 and

2), the true  $A_b$  value is considerably smaller than  $A_p$ , probably by at least one order of magnitude. In any case, however, the discrepancy is much smaller than with the former groups of membranes.<sup>18</sup>

If one now considers both groups of experiments together it becomes apparent that the disagreement between the  $A_p$  and  $A_b$  values is the smaller, the greater the porosity of the membranes and the higher their base exchange capacity; *i.e.*, the greater the charge density of the pore walls. We hope to be able to discuss in a subsequent publication the probable significance of this regularity which obviously must be closely correlated to the basic geometrical and electrical structure of the membranes.

#### IV

The attempt to verify the Teorell, Meyer-Sievers theory as applied to a variety of porous membranes by the comparison of selectivity constants arrived at on the basis of two different, experimentally independent methods has failed completely. In this section we shall discuss briefly the tentative conclusions which can be drawn from our experimental results concerning the Teorell, Meyer-Sievers theory. The conclusions must be based upon these two facts. On the one hand the theory predicts in a semiquantitative way the shape of the potential-concentration curves on the basis of clear and well defined assumptions; it therefore most likely contains at least a considerable element of basic or at least formal agreement with the reality. On the other hand, the  $A_p$  and the  $A_b$  values which should be identical according to the theory do not show any regular and reasonable agreement. This can indicate one of several possibilities, foremost amongst them that the attempt to measure  $A_b$  values is basically erroneous; or that the theory is basically erroneous; or that the theory, though at least formally correct in some respects (as far as the  $A_p$  values are concerned), is not based on a true, but on a one-sided, fictitious picture of the physical facts. It therefore may not permit an application which goes beyond the establishment of formal  $A_p$  values.

Of these three main possibilities the first one seems least likely, the theory being founded on the very assumptions which coincide with the definition of our  $A_b$  selectivity constant. For the above given reasons we are reluctant to accept the second possibility, namely, that the whole theory is basically erroneous. The greatest inherent probability seems to lie with the third of the above mentioned possibilities, namely, that the theory is built on a basis which though partially correct, neglects some essential features of the real physical

<sup>18</sup> It is interesting to note that there is no parallelism of the change of  $A_p$  and  $A_b$  with changes in water content. When the water content changed from 17 to 62 per cent,  $A_p$  decreases by a factor of 93, whereas  $A_b$  decreases by a factor of only 4.2. This is apparently not accidental, for in an analogous experiment with a set of membranes of varying porosity prepared from oxidized collodion, the same trend was found.

situation. This may have something to do with the rather formal character of the Teorell, Meyer-Sievers theory, which does not take into account any structural factors.

In a subsequent publication we shall try to integrate the outlined facts and conclusions with the results of earlier studies on collodion membranes. In this way we hope to move towards a more correct and more concrete picture of the geometrical and electrical structure of porous membranes.

#### SUMMARY

1. The Teorell, Meyer-Sievers theory characterizes the electrochemical behavior of membranes by their selectivity constant " $A_p$ " which is derived conventionally from concentration potential measurements at various concentration levels. The selectivity constant may, however, be derived also from entirely independent, different experimental data, namely base exchange studies. The constants arrived at in this second way are designated as " $A_b$ ." The selectivity constants derived by these two methods must be in reasonable, at least semiquantitative agreement if the basic assumptions of the theory are correct.

2. The selectivity constants  $A_p$  and  $A_b$  were determined for eleven different sets of membranes of different electrochemical activity and of different (8.2 to 80 volume per cent) water content.

3. The potentiometric selectivity constants  $A_p$  are in most cases several orders of magnitude greater than the corresponding  $A_b$  values. With membranes of great porosity and high electrochemical activity the  $A_b$  values approach at least in order of magnitude the  $A_p$  values.

4. It is concluded that the unexpectedly large discrepancy between the  $A_p$  and  $A_b$  values is due to some inherent weakness of the Teorell, Meyer-Sievers theory, most likely to its neglect of any structural factors.



# STUDIES OF THE INNER AND OUTER PROTOPLASMIC SURFACES OF LARGE PLANT CELLS

## II. MECHANICAL PROPERTIES OF THE VACUOLAR SURFACE

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The protoplasm of *Nitella* forms a thin layer surrounding a large central vacuole filled with sap. The outer region of the protoplasm, in contact with the cellulose wall and containing the chloroplasts, is motionless. It may form a rather rigid gel which may be caused by centrifugal force to peel off in long stiff strips containing the chloroplasts arranged in straight rows.

The protoplasm inside this layer is usually in motion and is separated from the liquid in the vacuole by a non-aqueous film which is too thin to be visible as a separate membrane, and hence is less than 0.5 micron in thickness. This film will be called *Y*.

When the sap is stained with neutral red<sup>1</sup> this non-aqueous film forms a sharply defined boundary between the deep red vacuole and the colorless protoplasm. It may be pushed violently in and out by the flowing protoplasm which often tends to thicken and become more lumpy under the influence of the stain.<sup>2</sup> Especially at the end of the cell where the stream of protoplasm flows across the end wall (where there are no chloroplasts) we may see that certain large lumps in the protoplasm push the non-aqueous film rapidly in and out (Fig. 1 *a*, *b*, and *c*), so that the observer expects to see it ruptured at any moment. In spite of this not a trace of dye appears in the protoplasm: this indicates that no such rupture occurs.

Mechanical disturbances of the film may occur at various places in the cell. The protoplasmic stream moves up one side of the cell and down the other and the two oppositely moving streams are separated by a surface having at its edges clearly visible white lines on opposite sides of the cell. These white lines are due to the absence of one or more of the rows of chloroplasts which run lengthwise from one end of the cell to the other. One may see particles in the stream approach a white line and come to rest in it for a time and

<sup>1</sup> A solution of 0.01 per cent of dye gives good results: 0.01 per cent of brilliant cresyl blue may also be used (both at pH 8).

<sup>2</sup> This is more striking after the cell has been in contact for several hours with a small volume of 0.01 per cent neutral red at pH 8 under a cover glass sealed at the edges with vaseline.



then return to the stream but very rarely do they cross into the other stream and go in the opposite direction.<sup>3</sup>

At first the white lines may run straight down the cell parallel to its long axis but later they become spirals and there is a corresponding twist of the surface<sup>4</sup> dividing the two oppositely flowing streams so that as one focusses through the cell the white line on the further side cuts that on the nearer side at a definite angle.

If the cell is in an upright position and the flow on the right side of the white line on the nearer surface is upward it is evident that if we could go around and view it from the other side the direction of flow would appear to be downward on the right side. Without performing this maneuver we need only move along the cell (focussing on the nearer surface) until the white line disappears from view in its spiral course

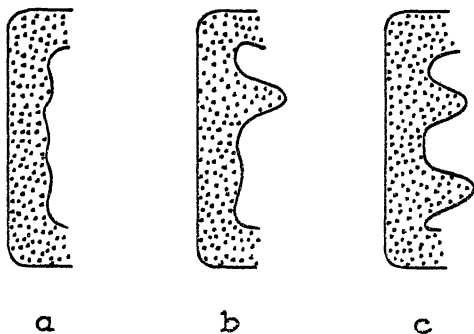


FIG. 1. Sketches at intervals of 5 seconds showing the conformation of the protoplasmic stream at the end of the cell (chloroplasts not shown). Semidiagrammatic.

around the cell: when it reappears further along on the nearer surface we see that the flow on the right side is now in the downward direction.

The protoplasm contains spheres of various sizes some of which are covered with spines, suggesting sphere crystals. These also occur in the sap and it seems evident that they must come in contact with the non-aqueous film.

In the sap we sometimes find gelatinous aggregates which revolve slowly since they extend clear across the vacuole and come in contact with the non-aqueous film on either side so that its motion is communicated to these masses.

The non-aqueous film undergoes a good deal of disturbance when the cell is

<sup>3</sup> When the protoplasm and chloroplasts are squeezed out of the cell (after cutting off one end) the location of the white line may often be detected by an apparent thickening (or thinning) of the cellulose wall.

<sup>4</sup> A model of this may be made by twisting a long narrow strip of cardboard so that each edge follows a spiral course: these edges then correspond to the white lines of the *Nitella* cell.

strongly plasmolyzed by sea water so that the large central vacuole breaks up into a series of smaller vacuoles, leaving the outer non-aqueous surface film, *X*, in its normal position as described in a former paper.<sup>5</sup> This involves a subdivision of the inner surface film, *Y*, yet there is no indication of the escape of dye from the vacuole when the cell is kept under continuous observation. In this process of division the film behaves as though it had true surface tension (the vacuole acts like a drop of oil in water) but we cannot say definitely whether it is a solid or a liquid film.

The question arises, how can the delicate non-aqueous surface layer of the vacuole survive so much mechanical disturbance.

It might be suggested that the protoplasm adjoining the vacuole is a water-in-oil emulsion (with the oil forming very thin films and the water in small drops only a micron or less in diameter) so that the non-aqueous surface film adjoining the vacuole would be relatively stable and if ruptured would be at once repaired.<sup>6</sup> It is questionable whether in that case we should find the high electrical capacity (1 microfarad per cm.<sup>2</sup>) observed in *Nitella*,<sup>7</sup> since the successive non-aqueous films might behave like condensers in series. It may also be asked whether such a layer would have the high degree of permeability to water which is found in *Nitella*.

It would seem that such a layer of emulsion could not be more than 2 or 3 microns thick for we see very small granules in the protoplasm, not more than 2 or 3 microns distant from the vacuolar surface, moving much more rapidly than larger granules and spheres equally distant from the vacuolar surface. If the small granules and the large spheres were in an emulsion of this sort we should not expect to see them moving at greatly differing rates.

Even if there were only a single non-aqueous film it is possible that ruptures might occur and be instantly repaired and thus escape detection. It has been suggested that the film is formed of substances which decrease surface tension and hence become trapped in the surface (for if they diffuse into it energy is required to remove them). Such substances might be available for the repair of the film.<sup>8</sup>

The properties of very thin non-aqueous layers between two aqueous systems are not understood. We have little to help us in dealing with this problem. Very thin films of liquids immiscible with water have been prepared

<sup>5</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1943-44, **27**, 139.

<sup>6</sup> In this case the surface would probably correspond more nearly with that of *Amoeba* and other protoplasmic surfaces which are subjected to motion.

<sup>7</sup> Blinks, L. R., *J. Gen. Physiol.*, 1936-37, **20**, 229; *Tr. Faraday Soc.*, 1937, **33**, 991. Curtis, H. J., and Cole, K. S., *J. Gen. Physiol.*, 1937-38, **21**, 189.

<sup>8</sup> Quick repair of a ruptured film is familiar to biologists in the well known experiment in which a crystal of copper sulfate is placed in a solution of potassium ferrocyanide.

but they do not seem to have a great degree of mechanical stability. Such films have been made by Danielli<sup>9</sup> and thinner ones have been prepared by Dean, Curtis, and Cole.<sup>10</sup>

The properties of the non-aqueous film surrounding the vacuole are of considerable interest. It is the chief seat of the resting potential of the cell which may amount to 100 mv. or more. If we consider the film to be 0.1 micron thick this would amount to an electrical pressure of 10,000 volts per cm.<sup>11</sup>

It would seem that this is largely due to the diffusion potential of KCl across *Y*. The sap contains about 0.05 M KCl but apparently there is very little K<sup>+</sup> in the aqueous layer of the protoplasm<sup>12</sup> (this layer may be called *W* for convenience). Hence we suppose that there is an outwardly directed concentration gradient<sup>13</sup> of KCl across *Y* and if the mobility of K<sup>+</sup> in *Y* were as much greater than that of Cl<sup>-</sup> as it is in *X* it might produce the observed resting potential of 100 mv. or more.<sup>12</sup>

It would also appear that the sudden loss of resting potential which occurs in stimulation is accompanied by a sudden increase in the permeability of *Y*.<sup>14</sup> The ability to produce action currents is lost when cells are leached in distilled water which appears to remove<sup>15</sup> an organic substance (or a group of such substances) called for convenience *R*. This can be recovered and replaced in the cell thus restoring irritability.<sup>16</sup> It can also be restored by blood,<sup>17</sup> by guanidine,<sup>18</sup> and by other substances.

It is also evident from previous work<sup>19</sup> that *Y* has selective permeability.

<sup>9</sup> Danielli, J. F., *J. Cell. and Comp. Physiol.*, 1935, **7**, 393.

<sup>10</sup> Dean, R. B., Curtis, H. J., and Cole, K. S., *Science*, 1940, **91**, 50. Dean, R. B., *Tr. Faraday Soc.*, 1940, **36**, 166. Dean, R. B., in Osterhout, W. J. V., Some models of protoplasmic surfaces, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, **8**, 62.

<sup>11</sup> Under normal conditions the pressure is presumably similar all over the surface so that there is little or no flow of current.

<sup>12</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1944-45, **28**, 23.

<sup>13</sup> By analogy with the behavior of the outer protoplasmic surface we may suppose that K<sup>+</sup> is the most important cation in this connection: accordingly the other cations are neglected for convenience in discussion. Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13**, 715. Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312.

<sup>14</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18**, 215; 1943-44, **27**, 61. Cole, K. S., and Curtis, H. J., *J. Gen. Physiol.*, 1938-39, **22**, 37.

<sup>15</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, **17**, 87.

<sup>16</sup> Osterhout, W. J. V., and Hill, S. E., *Proc. Soc. Exp. Biol. and Med.*, 1934-35, **32**, 715; *Science*, 1935, **81**, 418.

<sup>17</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1935-36, **19**, 423.

<sup>18</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1940-41, **24**, 7.

<sup>19</sup> Osterhout, W. J. V., *Bot. Rev.*, 1936, **2**, 283.

The dye does not escape because *Y* is not very permeable to  $H^+$  and in consequence the vacuolar sap is more acid than the protoplasm in *W*. Irwin<sup>20</sup> has shown that it is chiefly the difference between internal and external pH which determines the taking up of basic dyes, such as neutral red.

It is also of interest to note that *Y* differs in some respects from the corresponding non-aqueous layer *X* at the outer surface of the protoplasm.<sup>21</sup>

Furthermore, according to the results of Blinks<sup>22</sup> and of Curtis and Cole,<sup>23</sup> either *X* or *Y*, or both, must have a high electrical resistance and capacity.

Evidently, these non-aqueous surface layers present a variety of interesting problems to the physicist as well as to the chemist.

### Methods

The investigation was made on *Nitella flexilis* Ag. The cells were freed from neighboring cells and kept at  $15^{\circ}C. \pm 1^{\circ}C.$  in Solution A.<sup>24</sup> They were then placed (at about  $25^{\circ}C.$ ) in 0.01 to 0.05 per cent neutral red or brilliant cresyl blue (National Aniline and Chemical Co.) dissolved in distilled water. In some cases they were left in 10 ml. of the stain for a few minutes, then rinsed in distilled water, and observed. In other cases they were placed on a slide in the stain and covered with a cover glass,  $1 \times 2.5$  inches, with the edges sealed with vaseline. In this condition protoplasmic movement may continue for 2 or 3 days.

Generally speaking the youngest cells at the growing tip of the plant stain more deeply. This may indicate that their vacuolar sap is more acid since Irwin has shown<sup>20</sup> that staining of the vacuolar sap with brilliant cresyl blue and presumably with other basic dyes, such as neutral red, depends largely on this factor.

### SUMMARY

The vacuolar surface of *Nitella* is covered with a non-aqueous film too thin to be visible as a separate membrane. The motion of the protoplasm may subject this film to a good deal of mechanical disturbance.

<sup>20</sup> Irwin, M., *J. Gen. Physiol.*, 1925–28, **8**, 147; *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 125; 1931–32, **29**, 995.

<sup>21</sup> Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 391. Regarding the properties of *X* see Osterhout, W. J. V., *Physiol. Rev.*, 1936, **16**, 216; *Tr. Faraday Soc.*, 1937, **33**, 997; *J. Gen. Physiol.*, 1939–40, **23**, 171, 429; 1943–44, **27**, 91. Osterhout, W. J. V., and Hill, S. E., Some ways to control bioelectrical behavior, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1936, **4**, 43.

<sup>22</sup> Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 495; 1936–37, **20**, 229.

<sup>23</sup> Curtis, H. J., and Cole, K. S., *J. Gen. Physiol.*, 1937–38, **21**, 189. Cole, K. S., and Curtis, H. J., *J. Gen. Physiol.*, 1938–39, **22**, 37.

<sup>24</sup> Cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87.

Apparently this does not rupture the film for no dye escapes into the protoplasm as the result of such disturbance when the vacuolar sap is deeply stained with neutral red or brilliant cresyl blue.

When the deeply stained central vacuole breaks up into several smaller vacuoles, leaving the outer protoplasmic surface in its normal position, there is no evidence of the escape of dye into the protoplasm through the film surrounding the vacuole.

# DIFFERING RATES OF DEATH AT INNER AND OUTER SURFACES OF THE PROTOPLASM

## I. EFFECTS OF FORMALDEHYDE ON *NITELLA*

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When protoplasm dies it becomes completely and irreversibly permeable and this may be used as a criterion of death.

On this basis we may say that in *Nitella* where the protoplasm forms a thin layer surrounding the cell sap, death in formaldehyde may occur sooner at the inner than at the outer protoplasmic surface.

The outer and inner surfaces are non-aqueous layers which may be called respectively  $X$  and  $Y$ . Between these is an aqueous layer,  $W$ , which makes up the bulk of the protoplasm. Outside  $X$  is the cellulose wall which is so permeable that it may be neglected in the subsequent discussion.

The non-aqueous layers  $X$  and  $Y$  are the seats of the electrical potentials developed in the cell. When we apply formaldehyde we can tell which layer is being altered by following the changes in potential.

The potentials across  $X$  and  $Y$  are due to concentration gradients of electrolytes, chiefly of  $KCl$ .<sup>1</sup> The sap contains about 0.05  $M$   $KCl$  which appears to be much greater than the concentration in  $W$ . As a result there is a positive<sup>2</sup> diffusion potential of about 100 mv. across  $Y$ : this will be designated as  $P_y$ . The potential across  $X$  ( $P_x$ ) may be positive, negative, or zero. When the cell is bathed in tap water or in 0.00001  $M$   $KCl$ ,  $P_x$  appears to be very small.<sup>3</sup>

It is convenient to have a designation for the total potential ( $P_x + P_y$ ) with a fixed external solution and we may therefore call the potential across the protoplasm when the outside solution is 0.001  $M$   $KCl$  the "standard potential."

To measure the potential<sup>4</sup> the cell was placed on a paraffin block and solutions

<sup>1</sup> The influence of  $KCl$  on potential is predominant so that for convenience the effects of other salts may be neglected in this discussion.

<sup>2</sup> The potential is called positive when the positive current tends to flow from the sap across the protoplasm to the external solution.

<sup>3</sup> When the external solution is tap water or 0.00001  $M$   $KCl$  and  $X$  is made insensitive to  $K^+$  so that  $P_x$  disappears the total potential across the protoplasm does not fall off. Cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, 17, 87.

<sup>4</sup> The cells, after being freed from neighboring cells, stood in the laboratory at 15°C.  $\pm$  1°C. in Solution A (cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*,

were applied at three spots designated as *A*, *B*, and *C*. Under each of these spots there was an excavation in the paraffin block which held several milliliters of solution. Between each of these spots there was an excavation (2 cm. wide) filled with air: this sufficed to prevent short-circuiting. *A* and *B* were connected through a recording Einthoven galvanometer (with amplifier) to *C* which was in contact with 0.01 M KCl which kept the potential constant, approximately at zero.<sup>5</sup>

*Effects of Formaldehyde on the Inner Protoplasmic Surface, Y.*—When we apply 0.001 M KCl plus 0.2 M formaldehyde<sup>6</sup> the potential falls off and eventually disappears. Since *X* has little or no potential at the start the loss of potential must occur at *Y* and since it is irreversible we may conclude that *Y* becomes completely permeable.

When formaldehyde is applied there is at first a period<sup>7</sup> during which the curve falls a little (Fig. 1) or remains constant: this varies between 30 seconds and 2 minutes. After this the rise of the curve may be gradual throughout, as in Fig. 1, or it may begin in this way and suddenly rise.<sup>8</sup> The time required for the curve to reach zero<sup>9</sup> varies between 6 and 60 minutes.

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1933–34, 17, 87) for several days. Fig. 1 refers to cells in Lot B, Fig. 2 to cells in Lot A (cf. Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, 24, 312).

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, 21, 541). Temperature 20–26°C. Regarding the amplifier see the reference just cited.

<sup>5</sup> Two spots on the cell, *A* and *B*, were connected to a spot *C* through a recording galvanometer. At the end of the experiment *A*, *B*, and *C* were killed (in this order) by applying chloroform, which reduced the p.d. at each spot to zero.

It was then possible to ascertain the potential across the protoplasm at *A* and *B* at any previous point on the record on the assumption that *C* had remained constant up to that point (or by correcting for any change). If *C* had changed the amount of alteration could be detected because it would appear as a simultaneous change at *A* and *B* (in the same direction at both).

<sup>6</sup> This does not plasmolyze the cells used in these experiments. When the cells are dying we may see what has been called false plasmolysis. Cf. Osterhout, W. J. V., *Bot. Gaz.*, 1913, 55, 446.

<sup>7</sup> This appears to be due to a change in *X*. Some time is needed for penetration to *Y* (and the consequent rise of the curve) and this may be regarded as a latent period in respect to *Y*. Regarding latent periods in *Nitella* and other large plant cells see Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, 20, 13; 1939–40, 23, 569; 1940–41, 24, 311, 699; *J. Cell. and Comp. Physiol.*, 1941, 18, 129. Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, 22, 107.

<sup>8</sup> The sudden rise of the curve will be discussed in a subsequent paper.

<sup>9</sup> The time depends somewhat on the area of the cell covered by the solution. In these experiments about one-third of the cell was covered.

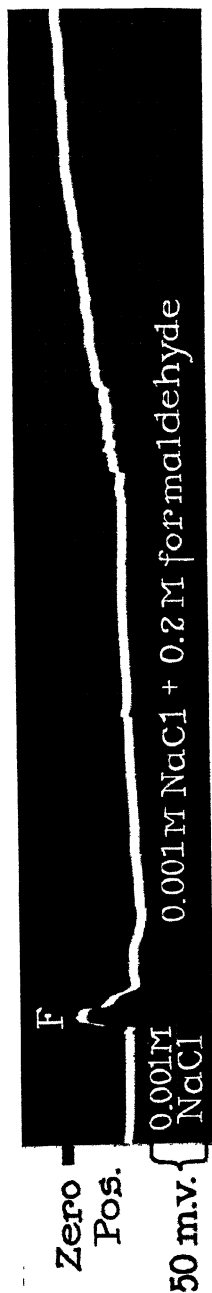


FIG. 1. The rise of the curve to zero shows the loss of potential under the influence of formaldehyde. At first the recorded region of the cell was in contact with 0.001 M NaCl. When the solution was removed this region was no longer in the circuit and the curve jumped to the "free grid" level marked "F." Then 0.001 M NaCl + 0.2 M formaldehyde was applied and the curve went back approximately to the previous level and after a slight dip began to rise.

The recorded spot was connected through a recording galvanometer to another spot in contact with 0.01 M KCl which kept the p.d. constant approximately at zero.

The cell was freed from neighboring cells and kept in Solution A for 29 days at  $15^{\circ}\text{C.} \pm 1^{\circ}\text{C.}$  and then placed in 0.001 M NaCl for 1 hour at  $25^{\circ}\text{C.}$  before the experiment was performed.

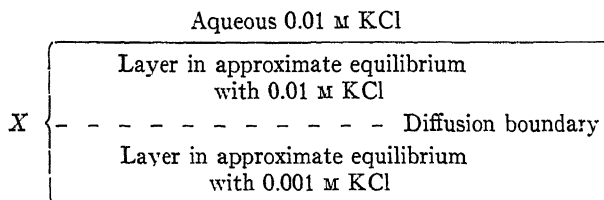
Time marks 15 seconds apart.



When the curve has risen to zero it usually remains there unless the external electrolyte is changed. But in some cases there is a temporary fall of the curve amounting to a few millivolts which may be explained on the ground that as  $Y$  becomes permeable (as shown by the previous loss of potential) KCl comes out of the sap and reaches  $X$ , thus setting up some potential which disappears<sup>10</sup> as KCl diffuses out through  $X$ .

The gradual rise in the curve in Fig. 1 indicates an increase in permeability of  $Y$  to electrolytes and especially to KCl and a loss of its potential<sup>11</sup> as KCl moves from the sap to the outside of  $Y$  (*i.e.*, into  $W$ ). When the outwardly moving KCl reaches  $X$  it can set up a noticeable positive potential if it advances with a sharp diffusion boundary and a sufficient concentration gradient but not if it enters  $W$  slowly and diffuses out through  $X$  as appears to be the case here.

*Effects of Formaldehyde on Ionic Mobilities in X.*—After  $Y$  becomes completely permeable, as shown by the total loss of standard potential, the outer protoplasmic surface,  $X$ , is still capable of responding to changes in electrolytes. This is shown by measurements of the concentration effect at  $X$ .<sup>12</sup> For example, if  $X$  has been standing for some time in contact with 0.001 M KCl which penetrates into  $W$  and this is replaced by 0.01 M KCl which begins to penetrate  $X$  we may have the situation shown in Scheme 1<sup>13</sup> (any possible phase boundaries are neglected).



SCHEME 1

<sup>10</sup> This is analogous to what frequently happens in the action current. Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18**, 215.

<sup>11</sup> In some cases the curve goes above zero while in contact with formaldehyde or just after its removal, indicating a negative potential at  $X$ .

<sup>12</sup> In this the immediate rise of the curve after applying the new solution shows that the effect is at  $X$ , since there is not sufficient time for the new solution to penetrate to  $Y$ .

<sup>13</sup> If a diffusion boundary and a diffusion potential exist in  $X$  the application of a suitable external solution will cause a change in potential due to the new diffusion boundary set up outside the old one. We may use the equation, putting the P.D. across the new diffusion boundary equal to the change of potential.

This gives a diffusion potential<sup>14</sup> of 28 mv. Previous experiments indicate<sup>15</sup> that in such cases we may calculate ionic mobilities by means of the usual equation which may be written (for 25°C.)

$$\text{P.D.} = 59 \frac{u_K - v_{Cl}}{u_K + v_{Cl}} \log \frac{a_1}{a_2}$$

where  $u_K$  and  $v_{Cl}$  are the mobilities of  $K^+$  and  $Cl^-$  in  $X$  and  $a_1 \div a_2$  is the ratio of activities of  $KCl$  in the outer and inner regions of  $X$ . Previous experiments<sup>16</sup> indicate that we may regard this as approximately equal to the ratio of concentrations in the external aqueous solutions; *i.e.*, as  $0.01 \div 0.001$ .

TABLE I

*Potential Differences, Relative Ionic Mobilities, and Partition Coefficients before and after Exposure for 7 Minutes to 0.001 M NaCl + 0.2 M Formaldehyde at about 25°C.*

Potassium effect 0.01 M KCl vs. 0.01 M NaCl		K concentration effect 0.01 M KCl vs. 0.001 M KCl		Na concentration effect 0.01 M NaCl vs. 0.001 M NaCl		Ratio of partition coefficients $S_{KCl} \div S_{NaCl}$		$u_K$ = mobility of $K^+$ in $X$		$u_{Na}$ = mobility of $Na^+$ in $X$	
Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
mv.	mv.	mv.	mv.	mv.	mv.						
33	0	28	20	33	25	17	1.4	2.81	2.03	3.54	2.47

For convenience we may put  $v_{Cl} = 1$  and we then have, before treatment with formaldehyde (Table I),

$$\text{P.D.} = 28 = 59 \frac{u_K - v_{Cl}}{u_K + v_{Cl}} \log \frac{0.01}{0.001}$$

whence

$$u_K = 2.81$$

This means that  $u_K \div v_{Cl} = 2.81$ .

Proceeding in the same way we find for the concentration effect of  $NaCl$ , *i.e.* 0.01 vs. 0.001 M  $NaCl$  a p. d. of 33 mv. whence  $u_{Na} \div v_{Cl} = 3.54$ .

After treatment with formaldehyde<sup>17</sup> we find for the concentration effect

<sup>14</sup> This potential is set up within a few seconds after the change in solution; *i.e.*, before there is time for the new solution to penetrate to  $Y$ .

<sup>15</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13**, 715.

<sup>16</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1942-43, **26**, 293.

<sup>17</sup> In this connection we may recall the fact that other organic substances can change mobilities in  $X$ . Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, **20**, 13, 685; 1937-38, **21**, 707; 1939-40, **23**, 171.

of KCl 20 mv. (Table I), whence  $u_K \div v_{Cl} = 2.03$ . For the concentration effect of NaCl we have 25 mv., whence  $u_{Na} \div v_{Cl} = 2.47$ .

This shows that  $X$  has not become completely permeable for in that case  $u_K$  would be practically equal to  $v_{Cl}$  (as in water) and the concentration effect would be nearly zero.<sup>18</sup> We find, however, that good concentration effects at  $X$  may be obtained more than an hour after  $V$  has become completely permeable (as shown by the complete loss of the standard potential across the protoplasm). We may, therefore, conclude that death arrives sooner at  $V$  than at  $X$ .

*Effect of Formaldehyde on Partition Coefficients As Related to the Potassium Effect.*—Under normal conditions  $X$  is able to distinguish electrically between  $Na^+$  and  $K^+$ . For example, when the cell has stood for some time in contact with 0.01 M KCl and this is replaced by 0.01 M NaCl a diffusion boundary is set up in  $X$  between KCl and NaCl. In consequence there is a diffusion potential which (Table I, p. 27) before treatment with formaldehyde amounts to 33 mv.

In previous papers<sup>19</sup> it has been customary to calculate partition coefficients by means of Henderson's equation which may be written for 25°C.

$$P.D. = 59 \frac{(U_I - V_I) - (U_{II} - V_{II})}{(U_I + V_I) - (U_{II} + V_{II})} \log \frac{U_I + V_I}{U_{II} + V_{II}}$$

where  $U_I = u_K c_K$ ,  $V_I = v_{Cl} c_K$ ,  $U_{II} = u_{Na} c_{Na}$ ,  $V_{II} = v_{Cl} c_{Na}$ . For convenience we put  $v_{Cl} = 1$  and  $c_{Na} = 1$  and insert the values already found (see Table I, p. 27); i.e.,  $u_K = 2.81$  and  $u_{Na} = 3.54$ . We then find if we put  $c_{Na} = 1$  and  $c_K = 17$  that the calculated P.D. is 33 which is the observed value. This means that if we define the partition coefficient  $S$  as the concentration in  $X$  divided by the concentration in the external solution we may put  $S_{KCl} \div S_{NaCl} = 17$  since the concentrations of KCl and NaCl in the external solutions are equal.

The treatment with formaldehyde lowers the value of the potassium effect<sup>20</sup> and may eventually reduce it to zero.<sup>21</sup> This is accompanied by a change in the ratio of partition coefficients. To calculate this change we may use the values for mobilities found after treatment with formaldehyde; i.e.,  $u_K =$

<sup>18</sup> In water the concentration effect of NaCl would be greater than that of KC<sup>1</sup> but in the freshly killed cell there is little difference because the sap comes out at once and as this contains 0.05 M KCl it cuts down the diffusion potential of NaCl.

<sup>19</sup> Cf. Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938-39, **22**, 139.

<sup>20</sup> The potassium effect may be completely abolished by an exposure for 1 minute to 0.1 M KCl + 0.2 M formaldehyde without making  $X$  completely permeable.

<sup>21</sup> After the loss of the potassium effect the concentration effects may persist for an hour or more.

2.03 and  $u_{\text{Na}} = 2.47$  (Table I, p. 27). We then find that if we put  $c_{\text{K}} = 1.4$  and  $c_{\text{Na}} = 1$  we obtain for the calculated p.d. a value of zero, as observed. This gives  $S_{\text{KCl}} \div S_{\text{NaCl}} = 1.4$  instead of 17, the value found before treatment.

Other cases where organic substances change partition coefficients have been observed in previous experiments with living cells<sup>22</sup> and with models.<sup>23</sup> This deserves further study.

*Restoration of the Potassium Effect.*—The potassium effect can also be removed by treatment with distilled water,<sup>24</sup> with acid,<sup>25</sup> and with alkali, and may be restored by the application of blood. An attempt was therefore made to restore the potassium effect after it had been removed by 0.1 M KCl + 0.2 M formaldehyde but the restoration was incomplete.<sup>26, 27</sup>

When the potassium effect is removed by distilled water the restoration by blood is usually complete.<sup>28</sup> This has been explained on the ground that the potassium effect depends on a substance (or group of substances) called for convenience *R* which is removed by distilled water and restored by the application of blood. When the restoration is incomplete, as after treatment with formaldehyde, it would seem that *X* has been altered so that even in the presence of *R* it cannot produce the complete potassium effect.

This throws an interesting light on variations in the potassium effect under normal conditions for it seems possible that substances are produced in metabolism which condition *X* so that even in the presence of a normal amount of *R* variations in the potassium effect occur. The values of the potassium effect under normal conditions range<sup>29</sup> from 26 mv. to 95 mv. Likewise the concentration effect of KCl and of NaCl varies and may depend on conditioning substances. The value for KCl (0.01 M vs. 0.001 M) ranges<sup>30</sup> from 22 mv. to 54 mv. The value for NaCl ranges from 20.9 mv.<sup>15</sup> to 34 mv.<sup>31</sup>

In the same way when a normal amount of *R* is present conditioning sub-

<sup>22</sup> Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1939–40, **23**, 171, 749.

<sup>23</sup> Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1943–44, **27**, 91.

<sup>24</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87, 105.

<sup>25</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 99.

<sup>26</sup> For example, if the potassium effect before treatment is 40 mv. and this disappears during exposure to formaldehyde the subsequent application of blood for 1 minute may restore it to 15 mv.

<sup>27</sup> Citrated whole sheep's blood was dried at 50°C. and an aqueous extract was made which had the same concentration as fresh blood diluted with 4 parts of water.

<sup>28</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1935–36, **19**, 423.

<sup>29</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715; 1938–39, **22**, 417; 1939–40, **23**, 171; 569.

<sup>30</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715; 1934–35, **18**, 987. Osterhout, W. J. V., and Hill, S. E., *Proc. Nat. Acad. Sc.*, 1938, **24**, 427.

<sup>31</sup> Unpublished results. See also footnote 30.

stances may influence the potential across  $Y$ . This appears to vary from 60 mv. to 200 mv.<sup>32</sup>

*Effects of Formaldehyde on Turgidity.*—The sap contains about 0.1 M chlorides plus some other substances which give an excess of osmotic pressure over the external solutions used in these experiments. As a result water enters the vacuole and produces a hydrostatic pressure which forces the protoplasm against the cellulose wall as an inner tube is forced against the outer casing of an automobile tire. The cell is then said to be in a turgid state. The pressure amounts to about 4 atmospheres.<sup>33</sup>

As a result a cell less than 1 mm. in diameter and 100 mm. long acquires so much mechanical rigidity that when grasped at one end and held in a horizontal position it does not bend perceptibly under its own weight. When the cell dies it becomes as flabby as a wet thread.

In order to test the effects of formaldehyde on the turgidity (*i.e.* rigidity) of the cell it was placed in a horizontal position over an excavation 2 cm. wide in the paraffin block. Thus the cell was supported only at the edges of the excavation. A ring of platinum wire weighing 0.175 gm. was suspended from the cell in the middle of the excavation which was filled with solution. As long as the cell retained its normal turgidity this weight did not cause the cell to sag perceptibly in the middle of the excavation but as soon as the turgidity fell off it began to do so.

When this excavation was filled with a solution of formaldehyde (which extended further along the cell so as to cover about one-third of its surface) the turgidity did not fall off although the potential had disappeared, indicating that  $Y$  had become completely permeable. It would seem, however, that  $X$  had not become completely permeable and it retained some of the osmotically active substances (not necessarily electrolytes effecting P.D.) so that turgidity was maintained. If  $X$  became permeable to these substances we should expect the turgidity to disappear. It can be made permeable by applying 0.001 M NaCl saturated with chloroform and when this was done the turgidity disappeared.

*Unlikeness of the Inner and Outer Protoplasmic Surfaces.*—It may seem surprising that the reagent which must pass through  $X$  in order to reach  $Y$  should produce death at  $Y$  sooner than at  $X$ . We know, however, from previous experiments that  $X$  and  $Y$  differ. Even when both are in contact with the same solution they behave differently. For example, when we set up the chain

Sap | Protoplasm | Sap

<sup>32</sup> Unpublished results. These values are found when the external solution is 0.001 M NaCl.

<sup>33</sup> The concentration of chlorides in the sap is about 0.1 M (about 0.05 M KCl + 0.05 M NaCl) which is osmotically equivalent to about 0.2 M cane sugar solution or to about  $0.2(22) = 4.4$  atmospheres. Hence it requires about 0.25 M cane sugar to plasmolyze. Smaller cells have less osmotic pressure than larger cells.

we obtain a value of 15 mv. which would be impossible if  $X$  and  $Y$  were identical in properties.<sup>34</sup>

It is, therefore, not surprising that differences are found when  $X$  and  $Y$  are in contact with unlike solutions.

We find differences in respect to the penetration of NaCl and other salts.<sup>35</sup> For example, when large cells are placed in 0.3 M NaCl the salt penetrates  $X$  more rapidly than  $Y$  and in consequence the osmotic pressure rises and  $W$  absorbs water from the vacuole.

We also find that certain organic substances can change the properties of  $X$  without making much change in the standard potential so that evidently there is not much change in  $Y$ .<sup>22</sup>

In the experiments with formaldehyde plus 0.001 M NaCl the potential across  $Y$  is very much greater than across  $X$  owing to the greater concentration gradient of KCl across  $Y$  and in consequence the electrical pressure across  $Y$  is much greater. If the potential across  $Y$  is 100 mv. and the thickness of  $Y$  is 0.1 micron the electrical pressure is 1000 volts per mm. It may be even greater since  $Y$  may be less than 0.1 micron in thickness.

It is not surprising that the increase in permeability should be linked to electrical pressure, since an increase in electrical pressure of 100 to 500 mv. can produce in *Nitella* the increase in permeability which leads to the action current. It must be remembered that under normal conditions the electrical pressure at any spot is approximately balanced by that at other spots so that little or no current flows.

It is also quite possible that the concentration of KCl may influence the result quite apart from its effect on electrical pressure.

In view of this it is desirable to test the effects of formaldehyde when  $X$  and  $Y$  are in contact with similar solutions. Since KCl plays an important rôle it was thought desirable to pay especial attention to it. As the sap contains about 0.05 M KCl a solution of 0.01 M KCl + 0.17 M formaldehyde was applied to  $X$ . The result<sup>36</sup> is shown in Fig. 2. Here it would appear that  $X$  loses its potential and becomes permeable more rapidly than  $Y$ <sup>37</sup> because the reagent reaches  $X$  first.

The curve shows the total potential across the protoplasm. At the start the external solution was 0.01 M KCl and the potential was approximately zero (Fig. 3). Then 0.01 M KCl + 0.17 M formaldehyde was applied and as  $X$

<sup>34</sup> Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, **11**, 391.

<sup>35</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1943-44, **27**, 139.

<sup>36</sup> The cells used in these experiments were from a different lot than that employed in the other experiments described in this paper.

<sup>37</sup> Cf. Osterhout, W. J. V., Some aspects of permeability and bioelectrical phenomena, in Molecular physics in relation to biology, *Bull. Nat. Research Council*, No. **69**, 1929, 170. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928-29, **12**, 355.

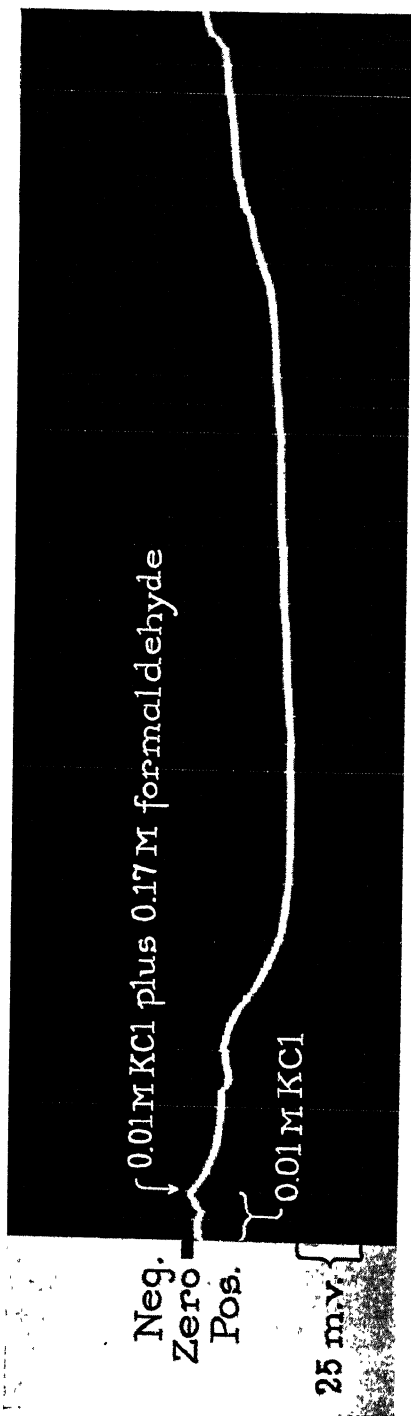


FIG. 2. The fall of the curve shows the increase of permeability of the outer non-aqueous protoplasmic layer  $X$ . The subsequent rise of the curve shows the increase of permeability of the inner non-aqueous protoplasmic layer  $Y$ . The protoplasm was in contact with 0.01 M KCl + 0.17 M formaldehyde which gave  $X$  at the start a negative potential of about 100 mv. which will be called  $P_x$ . At  $Y$  there was a positive potential of about the same value: this will be called  $P_y$ . The total potential,  $P_x + P_y$ , was equal to zero. Cf. Fig. 3.

Under the influence of formaldehyde the outer protoplasmic surface  $X$  became more permeable and  $P_x$  diminished so that the curve fell. Later the inner protoplasmic surface  $Y$  became more permeable and  $P_y$  diminished so that the curve rose. The recorded spot was connected through a recording galvanometer to a spot killed by chloroform in 0.01 M KCl which consequently had a p.d. value of zero.

The cell was freed from neighboring cells and kept in Solution A for 3 days at  $15^\circ\text{C.} \pm 1^\circ\text{C.}$  It was then kept for 1 hour at about  $22^\circ\text{C.}$  before the experiment was made. Time marks 5 seconds apart.

lost its potential more rapidly than  $Y$  the total potential became more positive and the curve fell. After about 1 minute the loss of potential at  $Y$  became faster than at  $X$  so that the total potential decreased and the curve began to rise.<sup>38-41</sup>

We may therefore conclude that death may occur at different rates in different parts of the cell and that this can be brought under experimental control.

*Potentials at  $X$  and  $Y$ .*—When the cell is treated with formaldehyde we often find that soon after the curve has risen to zero the concentration effect of KCl and of NaCl at  $X$  is the same as before treatment. We may therefore conclude

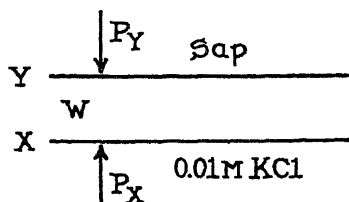


FIG. 3. Hypothetical diagram to illustrate potentials at the start of the curve in Fig. 2. The positive potential at  $Y$ , i.e.  $P_Y$ , is equal to the negative potential at  $X$ , i.e.  $P_X$ . When 0.01 M KCl plus 0.17 M formaldehyde is applied  $P_X$  begins to diminish, and the curve in Fig. 2 begins to fall. As formaldehyde penetrates to  $Y$  the value of  $P_Y$  falls off and the curve in Fig. 2 begins to rise.

that there has been little or no change in the permeability of  $X$  or in its reactions to electrolytes.

This simplifies our picture of the behavior of  $P_X$ . It has been stated (p. 23) that with 0.00001 M KCl outside the total potential ( $P_X + P_Y$ ) does not fall off when  $P_X$  is made to disappear so that we may put  $P_X = 0$  and  $P_Y = 100$  mv. If after treatment with formaldehyde we find that  $P_X + P_Y = 0$  it is probable

<sup>38</sup> Cf. Osterhout, W. J. V., Some aspects of permeability and bioelectrical phenomena, in Molecular physics in relation to biology, *Bull. Nat. Research Council*, No. 69, 1929, 170. The behavior of the curve varies with different cells; in some cases the drop is slower and less pronounced.

<sup>39</sup> Similar effects are obtained with 0.1 M KCl + 0.01 M HgCl<sub>2</sub>.

<sup>40</sup> This is quite the opposite of what happens when a reagent produces alteration at  $Y$  earlier than at  $X$  and the potential at  $Y$  falls after which KCl coming out and reaching  $X$  sets up a potential. In this case the potential first becomes more negative and then more positive. Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, 18, 215.

<sup>41</sup> If we apply 0.01 M KCl alone the curve remains for a long time at the same level and does not show the fall and rise seen in Fig. 2.



that the loss of potential and the change in permeability has been confined to  $Y$ .<sup>42,43</sup>

If the external solution is 0.00001 M KCl and  $P_x = 0$  we may say that the concentration of KCl in  $W$  is 0.00001 M if we assume that the p.d. is wholly due to KCl.<sup>44,45</sup>

This picture of KCl with a low concentration externally and in  $W$  and a relatively high concentration in the sap corresponds to what is observed with such dyes as brilliant cresyl blue and neutral red. When the concentration of dye is very low in the external solution it is very low in  $W$  but may be relatively high in the sap.<sup>46</sup>

If the concentration effect of 0.001 M vs. 0.01 M KCl is 33 mv. (a value commonly found in this lot of cells) we may write

$$31 = 59 \frac{u_K - v_{Cl}}{u_K + v_{Cl}} \log \frac{0.01}{0.001}$$

whence<sup>47</sup>  $u_K \div v_{Cl}$  in  $X = 3.54$ . Hence if we have 0.00001 M KCl in  $W$  and 0.01 M KCl outside  $X$  we may expect for the value of  $P_x$  (putting  $v_{Cl} = 1$ )

$$\begin{aligned} P_x &= 59 \frac{3.54 - 1}{3.54 + 1} \log \frac{0.01}{0.00001} \\ &= 99 \text{ mv.} \end{aligned}$$

This will have a negative sign so that the total potential will be  $P_y + (-P_x) = 100 - 99 = 1$  mv. which is the observed value.

<sup>42</sup> This appears to be the case during stimulation. Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1943-44, **27**, 61.

<sup>43</sup> Regarding a temporary increase in  $P_x$  in some cases see p. 26.

<sup>44</sup> This may be allowable as a first approximation.

<sup>45</sup> Brooks has reported relatively high concentrations of electrolytes in the protoplasm but these may be in organic combination and not in ionic form. Cf. Brooks, S. C., The intake of radioactive isotopes by living cells, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, **8**, 173.

<sup>46</sup> Regarding brilliant cresyl blue see Irwin (Irwin, M., *J. Gen. Physiol.*, 1925-28, **8**, 147; 1925-26, **9**, 235, 561; 1926-27, **10**, 75, 927; 1927-28, **11**, 123; 1928-29, **12**, 147, 407; *Proc. Soc. Exp. Biol. and Med.*, 1926-27, **24**, 425; 1927-28, **25**, 127; 1928-29, **26**, 125, 135; 1931-32, **29**, 993, 995, 1234). The behavior of the dye depends on pH which does not seem to be the case with KCl (cf. Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18**, 967).

<sup>47</sup> A similar calculation for  $Y$  gives

$$100 = 59 \frac{u_K - v_{Cl}}{u_K + v_{Cl}} \log \frac{0.05}{0.00001}$$

whence  $u_K \div v_{Cl}$  in  $Y = 2.7$

When we place sap outside  $X$  we find as a rule that the total potential<sup>48</sup> is about 15 to 20 mv. negative so that  $P_x = 115$  to 120 mv. negative when  $P_y = 100$ . This is to be expected since we may write (putting  $u_K = 3.54$  and  $v_{Cl} = 1$ )

$$P_x = 59 \frac{3.54 - 1}{3.54 + 1} \log \frac{0.5}{0.00001}$$

$$= 122 \text{ mv.}$$

These values<sup>48</sup> are given merely by way of illustration: they may vary with each lot of cells.<sup>49</sup>

#### DISCUSSION

These results have a direct bearing on some fundamental questions. Death makes the protoplasm completely and permanently permeable so that this may be used as a criterion of death. On this basis we may say that death occurs sooner at the inner non-aqueous protoplasmic surface than at the outer. The inner surface is the chief seat of the positive potential and when this potential disappears we may regard the inner surface as completely permeable.

When the outer surface becomes completely permeable to electrolytes it can no longer distinguish electrically between different concentrations of the same salt. But this may not occur for an hour or more after the inner surface becomes completely permeable.

Before this, however, a change occurs in  $X$  which leads to the disappearance of the potassium effect. This change is due chiefly to alterations in the partition coefficients of KCl and NaCl in  $X$ . Before exposure to formaldehyde the ratio of partition coefficients (*i.e.*,  $S_{KCl} \div S_{NaCl}$ ) is 17; after exposure it is 1.4 (Table I, p. 27). Hence the concentration of  $Na^+$  in  $X$  approaches that of  $K^+$  and the potassium effect falls off.

It is evident that the properties of the non-aqueous protoplasmic surfaces

<sup>48</sup> The relatively low concentration of KCl provisionally suggested for  $W$  is in line with experiments on basic dyes: no matter how great the concentration of dye in the sap the concentration in  $W$  is always very low. Brooks (Brooks, S. C., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, **8**, 171) has stated that relatively high concentrations of radioactive salts may occur in the protoplasm (as compared with the sap) but it seems possible that they may be in combination with organic substances: there is no proof that they are present in ionic form in the protoplasm at high concentrations.

<sup>49</sup> For higher values of the concentration effect of KCl see Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13**, 715; Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, **21**, 541; *Proc. Nat. Acad. Sc.*, 1938, **24**, 312. These correspond to higher concentrations of KCl in  $W$ . For higher values of  $P_x$  see Osterhout, W. J. V., *J. Gen. Physiol.*, 1943-44, **27**, 61.

can be brought under experimental control to a considerable degree. This deserves further study.

#### SUMMARY

When protoplasm dies it becomes completely and irreversibly permeable and this may be used as a criterion of death. On this basis we may say that when 0.2 M formaldehyde plus 0.001 M NaCl is applied to *Nitella* death arrives sooner at the inner protoplasmic surface than at the outer.

If, however, we apply 0.17 M formaldehyde plus 0.01 M KCl death arrives sooner at the outer protoplasmic surface.

The difference appears to be due largely to the conditions at the two surfaces. With 0.2 M formaldehyde plus 0.001 M NaCl the inner surface is subject to a greater electrical pressure than the outer and is in contact with a higher concentration of KCl. In the other case these conditions are more nearly equal so that the layer first reached by the reagent is the first to become permeable.

The outer protoplasmic surface has the ability to distinguish electrically between  $K^+$  and  $Na^+$  (potassium effect). Under the influence of formaldehyde this ability is lost. This is chiefly due to a falling off in the partition coefficient of KCl in the outer protoplasmic surface.

At about the same time the inner protoplasmic surface becomes completely permeable. But the outer protoplasmic surface retains its ability to distinguish electrically between different concentrations of the same salt, showing that it has not become completely permeable.

After the potential has disappeared the turgidity (hydrostatic pressure inside the cell) persists for some time, probably because the outer protoplasmic surface has not become completely permeable.

## DIFFERING RATES OF DEATH AT INNER AND OUTER SURFACES OF THE PROTOPLASM

### II. NEGATIVE POTENTIAL IN *NITELLA* CAUSED BY FORMALDEHYDE

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In *Nitella* the normal potential is positive,<sup>1</sup> amounting to about 100 mv. Under the influence of formaldehyde it may gradually disappear, as described in a former paper.<sup>2</sup>

In the present paper it will be shown that there may also be a sudden and striking access of negative potential.

This is illustrated in Fig. 1 which shows the record of a spot *A* which was connected to another spot *C* on the same cell through a recording galvanometer.<sup>3</sup> At first *A* was in contact with 0.001 M NaCl. This was replaced by 0.001 M NaCl plus 0.2 M formaldehyde which caused a drop in the curve, indicating an increase in positive potential. This may be due to the loss of a small negative potential present at the start at the outer non-aqueous protoplasmic surface (called *X*).

After a short period<sup>4</sup> which may be due to the time required for formaldehyde

<sup>1</sup> The potential is called positive when the positive current tends to flow from the sap across the protoplasm to the outside solution.

<sup>2</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1944-45, **28**, 23.

<sup>3</sup> The cells, after being freed from neighboring cells, stood in the laboratory at 15°C.  $\pm$  1°C. in Solution A (cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, **17**, 87) for several days. They belonged to Lot B (cf. Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312).

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, **21**, 541). Temperature 20-26°C. Regarding the amplifier see the reference just cited.

Two spots on the cell, *A* and *B*, were connected to a spot *C* through a recording galvanometer. At the end of the experiment *A*, *B*, and *C* were killed (in this order) by applying chloroform which reduced the p.d. to zero.

It was then possible to ascertain the potential across the protoplasm at *A* and *B* at any previous point on the record on the assumption that *C* had remained constant up to this point (or by correcting for any change). If *C* had changed the amount of alteration could be detected because it would appear as a simultaneous change at *A* and *B* (in the same direction at both).

<sup>4</sup> Regarding latent periods in *Nitella* and other large plant cells see Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, **20**, 13; 1939-40, **23**, 569; 1940-41, **24**, 311, 699; *J. Cell. and Comp. Physiol.*, 1941, **18**, 129. Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, **22**, 107.



FIG. 1. Shows an abrupt rise near the end of the curve indicating a sudden access of negativity. This occurred when *Y* had become completely permeable to electrolytes as shown by the rise of the curve to zero.

At first the recorded spot was in contact with 0.001 M NaCl. When the solution was removed the spot was no longer in the circuit and the curve jumped to the free grid level "F." Then 0.001 M NaCl plus 0.2 M formaldehyde was applied and the curve resumed its former level and after a dip it rose slowly to zero after which the abrupt rise took place. The recorded spot was connected through a recording galvanometer to another spot in contact with 0.01 M KCl which kept the p.d. constant approximately at zero.

The cell was freed from neighboring cells and kept in Solution A for 33 days at  $15^{\circ}\text{C.} \pm 1^{\circ}\text{C.}$  and then placed in 0.001 M NaCl for an hour at  $25^{\circ}\text{C.}$  before the experiment was performed.

Time marks 15 seconds apart.

to penetrate through the aqueous layer of the protoplasm (called *W*) to the inner non-aqueous protoplasmic surface (called *Y*) the curve began to rise, indicating a loss of the positive potential at *Y*.

The positive potential at *Y* is due to a concentration gradient of electrolytes, mostly of KCl, across *Y*. The concentration of KCl in the sap is about 0.05 M but in *W* it appears to be relatively low.<sup>2</sup>

As *Y* becomes more permeable KCl moves out into *W* thus lessening the concentration gradient and the potential across *Y*. This is a slow process, as shown by the slow rise of the curve. In some cases KCl reaches *X* very gradually and apparently diffuses out through *X* so that the concentration gradient of KCl across *X* is always small and any positive potential across *X* due to KCl is also small.<sup>5</sup>

It thus appears that the permeability of *Y* to KCl increases under the influence of formaldehyde and causes a gradual loss of potential and that as the process continues *Y* becomes permeable to certain other substances which diffuse outward and produce negative potential when they arrive at *X*. If this negative potential is a diffusion potential it is evident that in general the anions of these substances must have a higher mobility than the cations in *X*.

It is not surprising that the final step in the process (*i.e.* the step which releases the negativating substances) should involve a sudden change, as shown by the abrupt rise of the curve.

If the substances producing negative potential at *X* exist in the sap they must set up much less negative potential against *Y* than against *X* but it is probable that they do not exist as such in the sap but are formed in *W* by a combination between formaldehyde and certain substances coming from the sap. (Such results have been found only in experiments with formaldehyde although other substances can cause *Y* to lose its potential.)

These substances appear to diffuse out through *X*, causing the curve to fall. The fact that it falls slowly indicates that *X* has not become completely permeable (Fig. 2).

At this time *X* may show some potassium effect; *i.e.*, some change of potential when 0.01 M KCl is replaced by 0.01 M NaCl. Also good concentration effects may be obtained now and later on with NaCl and KCl as described in a former paper.<sup>2</sup> This also shows that *X* has not become completely permeable.

It may be added that if chloroform<sup>6</sup> is applied at this time so that *X* becomes completely permeable the negative potential disappears and the curve falls to zero.

<sup>5</sup> Occasionally it may be noticeable, as shown in a former paper (see footnote 2). It may be larger in the action current where KCl comes out rapidly, *cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18**, 215.

<sup>6</sup> A saturated solution of chloroform in 0.001 M NaCl.

The idea that organic substances in *W* may set up a potential finds support in experiments on *Halicystis*. Here the external solution is nearly the same as the sap but the potential amounts to about 68 mv. positive.<sup>7</sup> This is changed to about 40 mv. negative by treatment with ammonia.<sup>8</sup> It would seem that organic substances are responsible for these potentials and they are probably produced in *W*.

These facts lead us to assume that the sudden rise of the curve is due to an effect on *X* caused by organic substances passing out from the sap through *Y* and combining with formaldehyde in *W*.

As the permeability of *Y* increases under the influence of formaldehyde it eventually becomes completely permeable to electrolytes so that it loses all of its potential. It would seem that after it becomes permeable to KCl the final change may make it permeable to all sorts of substances, leading to the abrupt rise of the curve as in Fig. 1. It is not surprising that the final step in this process may be sudden, as in Fig. 1, because *Y* is a thin non-aqueous film between two aqueous solutions and is subject to the forces of surface tension and therefore to sudden rupture when a certain stage is reached. But even without such a rupture a sudden increase in permeability is conceivable.

Such a sudden change may occur earlier in the process and this is especially apt to happen when solutions are changed; e.g., from 0.001 M NaCl plus formaldehyde to 0.001 M NaCl (Fig. 2). In this case the mechanical disturbance may play a part but there are also changes in osmotic pressure and in chemical composition.

Some other suggestions may now be considered.

(1) It might be thought that the substances moving out from the sap cause the negativity by increasing the negativating action of the NaCl outside of *X*. But the NaCl moving out from the sap and diffusing out through *Y* to *X* might be expected to create an opposing potential at *X*. There is no increase in the mobility of Na<sup>+</sup> and consequently of its negativating action at this time: this is shown by measurements of the concentration effect of NaCl (e.g. by substituting 0.01 M for 0.001 M NaCl in contact with *X*). Moreover we find the same effect when we apply formaldehyde dissolved in distilled water instead of in 0.001 M NaCl.

(2) It might be suggested that the gradual rise of the curve in Fig. 1 is due to gradual growth of negative potential at *X* due to the outward movement of substances from the sap which after combining with formaldehyde in *W* come in contact with *X*. In the meantime the potential at *Y* might suffer little or no change until it suddenly disappeared, producing a sudden rise of the curve.

Such a sudden rise occurs in the action current<sup>9</sup> but the resulting curve

<sup>7</sup> Blinks, L. R., *J. Gen. Physiol.*, 1932-33, **16**, 147.

<sup>8</sup> Blinks, L. R., *J. Gen. Physiol.*, 1933-34, **17**, 109.

<sup>9</sup> Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18**, 215.

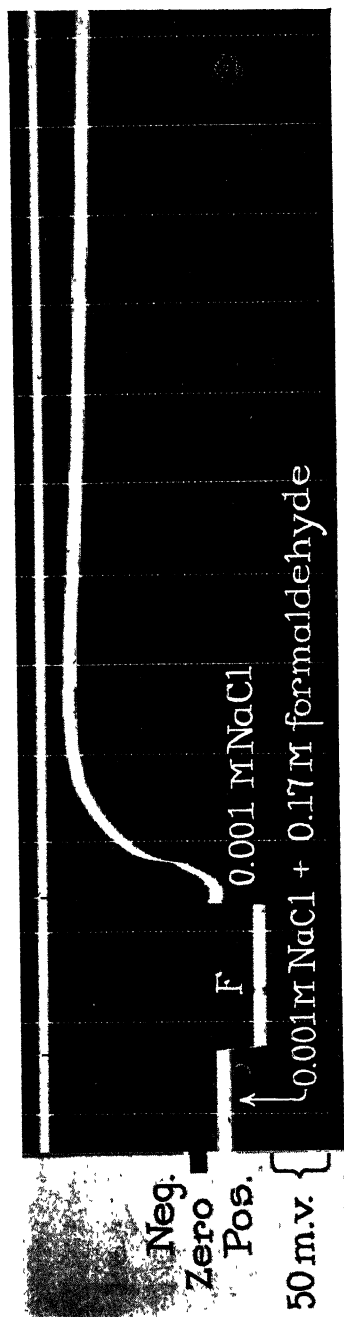


FIG. 2. Shows an abrupt rise of the curve indicating a sudden access of negativity. This occurred before  $Y$  had become completely permeable since it took place before the curve had risen to zero.

At first the recorded spot was in contact with  $0.001\text{ M NaCl}$  and the curve was  $59\text{ mv.}$  below zero. Then the spot was placed in contact with  $0.001\text{ M NaCl} + 0.17\text{ M formaldehyde}$  and the curve rose slowly  $27\text{ mv.}$  before the beginning of the record shown here. When the solution was removed the spot was no longer in the circuit and the curve jumped to the free grid level "F." Then  $0.001\text{ M NaCl}$  was applied and the curve rose abruptly  $110\text{ mv.}$  and then began to fall slowly.

The recorded spot was connected through a recording galvanometer to another spot in contact with  $0.001\text{ M KCl}$ ; at this spot the p.d. remained constant.

The cell was freed from neighboring cells and kept in Solution A for 2 hours at about  $25^{\circ}\text{C.}$  before the experiment was made. Time marks 15 seconds apart.



differs from that seen in Fig. 1, since it has a sharp point of inflection at the apex which appears to be due to the outward movement of KCl causing the curve to rise and then to fall as KCl comes in contact with *X*. The curve in Fig. 1 is just what would be expected if substances with relatively rapid anions were diffusing out to *X*.

If a gradual growth of negative potential at *X* occurred we might expect it sometimes to carry the curve above zero before the sudden change but this does not occur. When, as often happens, there is no sudden change<sup>2</sup> but only a slow rise of the curve to zero it is natural to suppose that this is due to a gradual increase in the permeability of *Y* and this applies also to the first part of the curve in Fig. 1.

In some cases the abrupt rise of the curve shows a loss of potential greater than the total potential at the start as in Fig. 2. Evidently this cannot be entirely due to loss at *Y*.

#### SUMMARY

A previous paper showed that when the inner protoplasmic surface has lost its potential under the influence of formaldehyde the outer surface can still respond to changes in the concentration of electrolytes.

The present paper indicates that after the inner surface has lost its potential there may be a sudden development of negative potential at the outer surface due to substances coming out of the sap and combining with formaldehyde.

# ACTION OF POTASSIUM AND NARCOTICS ON RECTIFICATION IN NERVE AND MUSCLE\*

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## INTRODUCTION

The characteristics of the cell membrane seem to be of primary importance in the case of both conduction and contraction. Among the membrane qualities recently discovered is electrical rectification; *viz.*, the ability of the membrane to permit electrical current to pass more easily outward than inward (Cole and Curtis, 1941; Guttman and Cole, 1941). The present paper constitutes an attempt to study the mechanism involved in tissue rectification by observing the effect of variation of the ionic medium bathing the cells.

It will be shown that the potassium ion decreases rectification in frog nerve and muscle and that this effect is reversible. It will also be shown that various narcotics, *e.g.* chloroform, isoamyl carbamate, veratrine sulfate, also have the ability to decrease the rectifying property of frog nerve and muscle and that lack of calcium, excess calcium, or barium, have no such effect.

While it has been known since 1941 that electrical rectification is exhibited by the single nerve fiber of the squid, it is of interest that this property can also be demonstrated in whole nerve and in whole muscle (*cf.* Katz, 1942), and moreover in the classical preparations long used in physiological laboratories; *i.e.*, frog sciatic nerve and frog sartorius muscle. A few experiments carried out in Woods Hole in the summer of 1941 indicate that rectification occurs also in the single nerve fiber of *Ommastrephes illecebrosus*, the northern squid. Whether the rectifying property is a general property present in the plasma membrane of all types of cells, or is confined to nerve and muscle cells, where a propagated type of activity occurs, is something which should be worth investigation.

## *Preliminary Experiments*

Preliminary experiments were carried out in Woods Hole in the summer of 1940 and 1941. There the material used was the giant nerve fiber of the hindmost stellar nerve of the squid, *Loligo pealii*, and also of the northern squid, *Ommastrephes illecebrosus*.

\* Aided by a grant from the Permanent Science Fund of the American Academy of Arts and Sciences.

Immediately after dissection the axon was threaded through a glass U tube. Then the U tube was suspended by means of a glass rod cemented to it in such a way that one end of the fiber dipped into sea water, where it remained in good condition for many hours and the other end into a 0.48 M KCl solution twenty times as concentrated with respect to the K ion as sea water or else into 0.52 M KCl approximately isosmotic with sea water, which injured that end. Experimental solutions were then substituted for the sea water at the uninjured end. In previous experiments (Guttman and Cole, 1941) the interelectrode region (which was about 1 cm. long) lay in oil, but in these experiments it was found that many experimental difficulties, *e.g.* creeping of the electrode levels, were alleviated if air was substituted for the oil. The measuring circuit was much the same as that described in a previous paper (Fig. 1, Guttman and Cole, 1941) except that an amplifier and cathode ray oscillograph were substituted for the galvanometer previously used as a detector in the D.C. Wheatstone bridge circuit.

In these preliminary experiments only relative values of rectification were obtained. Degree of deflection, *i.e.* offbalance of the stationary spot of the oscillograph was plotted against the amount of the voltage imposed upon the fiber for currents flowing first in one direction and then in the other (anodal and cathodal currents).

### *Material and Methods*

The sciatic nerve and the sartorius muscle of *Rana pipiens* were used in these experiments. (All frogs used were winter frogs.) In the case of the sciatic nerve, the sheath was either partially removed or else slit with Swiss watchmaker forceps while in Ringer's solution under a binocular microscope. This was done to insure easy penetration of the chemical agents used.

The amphibian Ringer's solution used was buffered to pH 7.4 with sodium bicarbonate. Except where otherwise noted all solutions used were isosmotic with Ringer's solution. However, it was found that slight variations in osmotic pressure had no appreciable effect upon the amount of rectification observed.

After dissection the tissue was mounted in a chamber similar to that used in a previous paper (Guttman, 1940). It consisted (Fig. 1) of a box containing paraffin, in which were imbedded at right angles two U shaped glass tubes, *BB'* and *CC'* and a blind glass tube, *A*. The distance (outside measurement) between *A* and *C* was about 4 cm.

Tube *A* was filled with Ringer's solution. Tube *BB'* was filled either with Ringer's or some test solution, whose effect upon the tissue was being investigated. Tube *CC'* was usually filled with isosmotic KCl (0.116 M).

Two glass supports (*S*), imbedded in the paraffin, suspended the nerve or muscle in such a way that one end dipped into the Ringer's solution at *A*, the middle dipped into Ringer's solution or test solution at *B*, and the other end dipped into the isosmotic KCl solution at *C*, which served to injure that end.

A moist chamber (*M.C.*), whose edge was smeared with vaseline, fitted into a circular groove in the paraffin and enclosed the tissue, portions *A*, *B*, and *C* of the tubes,

and the glass supports (*S*). The glass supports were coated with vaseline and the surface of the paraffin was kept dry to avoid creeping. One calomel half cell made contact with the tissue at *B'* and another at *C'*.

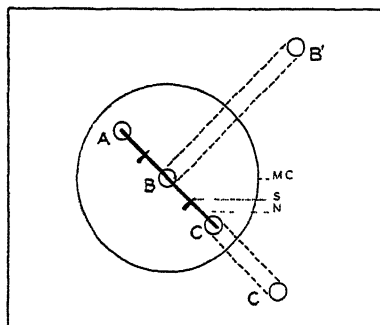


FIG. 1. Mounting chamber for measuring electrical rectification and resting potentials of frog nerve or muscle, top view. Dotted line indicates portion of glass tubing below the surface of solid paraffin. *A*, blind glass tube containing Ringer's solution; *BB'*, glass U tube containing Ringer's or test solution; *CC'*, glass U tube containing isosmotic KCl; *S*, glass support; *M.C.*, moist chamber; *N*, nerve or muscle.

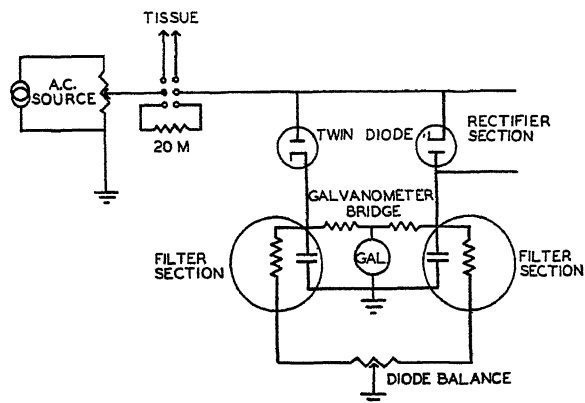


FIG. 2. Measuring circuit. Explanation in text.

### *Apparatus*

The a. c. tissue rectification measuring circuit as used in the main body of experiments is shown in Fig. 2. The circuit impresses a sinusoidal voltage (of 1 volt, root mean square) across the tissue and the measuring circuit in series. When the tissue exhibits rectifying properties the voltage drop across the tissue differs for the positive and negative half cycles of the applied *E. M. F.*, due to a greater resistance of the tissue to current flow in one direction. The measuring circuit consists of a twin diode, each

section being used to rectify one polarity of the applied wave. A filter circuit employed with each diode section develops a D. C. voltage nearly equal to the peak value of the applied E. M. F. Since the positive and negative half cycles of the wave are of unequal amplitude due to partial rectification in the tissue, the D. C. output voltages of the diode sections will also be unequal. The difference between these two voltages causes the galvanometer to deflect in proportion to the degree of rectification.

The instrument is calibrated by introducing a small D. C. voltage at the tissue test terminals. This shifts the axis of the A. C. wave, simulating partial rectification. A calibration plot is then made of galvanometer deflection vs. percentage rectification. Since the peak voltage of the 1 volt root mean square applied sine wave is  $\frac{1}{0.707}$  or 1.414 volts we can get a measure of the rectification by comparing the D. C. component of the rectified wave with this figure. Thus

$$\text{Per cent rectification} = \frac{V}{1.414} \times 100 \text{ per cent}$$

where  $V$  equals the D. C. component in volts or "shift" in the A. C. axis of the sine wave. This method is sufficiently accurate for the small percentages of rectification encountered in this work. For high percentages a wave form factor would have to be included in the formula.

Although this measuring circuit was quite satisfactory and was very simple, an improvement in the sensitivity can be obtained by replacing the galvanometer with a vacuum tube voltmeter. This is contemplated in future experiments.

Frequency was varied from 30 cycles to 10,000 cycles without perceptible differences in results, by means of a General Radio 713 B beat frequency oscillator. Most of the data were taken at 60 cycles.

Resting potentials were measured at the calomel half cells by means of a potentiometer and galvanometer.

The rectification varied between 1 and 3 per cent in the experiments with frog material. The experimental error was estimated to be below 10 per cent. Rapidity of making measurements before the tissue had a chance to change resistance or the apparatus to become unbalanced was a means of obtaining significant measurements of small amounts of rectification. On reversing the current, similar rectification was always obtained.

## RESULTS

Eighteen preliminary and twenty-seven subsequent experiments were done in all. All figures represent typical results.

*Effect of Potassium upon Tissue Rectification.*—In all cases potassium decreases tissue rectification, the degree of the effect depending upon the concentration. This effect is either partially (Fig. 3 A) or completely (Fig. 3 B) reversible, depending upon the concentration used and the length of time the solution is permitted to act.

In the experiments represented by Fig. 3, the reference end of the tissue was placed in isosmotic KCl (0.116 M) and the experimental end treated alter-

nately with Ringer and KCl solution, to show the effect of KCl upon the tissue. In another type of experiment, a slightly different technique was used; *viz.*, the reference end of the tissue remained in Ringer's solution and the experimental portion was referred to this untreated end. In such a case, when both portions were in Ringer's solution no rectification was observed, but when the experimental end was treated with potassium, rectification appeared.

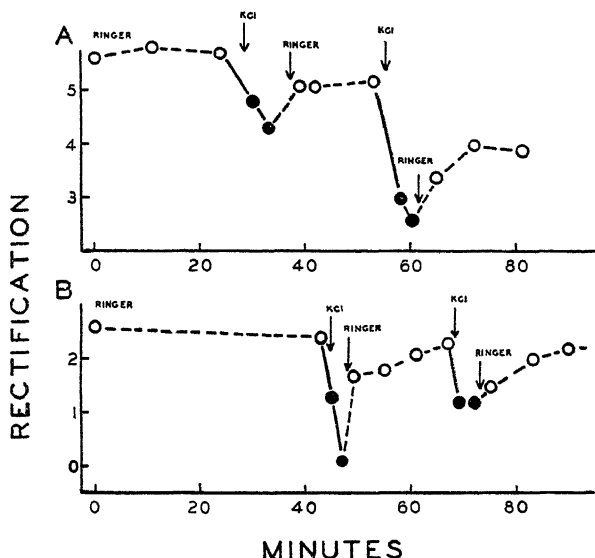


FIG. 3. Effect of potassium upon rectification in A, sartorius muscle and B, sciatic nerve of *Rana pipiens*. Rectification in arbitrary units directly proportional to D. C. component of the current passing through the tissue *vs.* time in minutes. "Ringer" indicates that one electrode region was immersed in Ringer's solution and the other in isosmotic KCl (0.116 M). "KCl" indicates that both electrode regions were immersed in isosmotic KCl. A decrease in resting potential accompanied every decrease in rectification. Both rectification and resting potential changes are reversible, wholly or in part.

*Narcotics Decrease Rectification.*—Narcotics are another group of substances which are known to affect the membrane profoundly. As might have been expected, narcotics, like potassium, reversibly decrease rectification. This was shown a few years ago in the case of cocaine and the single nerve fiber of the squid (Guttman and Cole, 1941). It can also be demonstrated for frog sciatic and sartorius using chloroform, veratrine sulfate and isoamyl carbamate (Fig. 4).

*Excess Calcium and Barium, and Lack of Calcium Do not Affect Rectification.*—Because of the very suggestive effect of low calcium in initiating spontaneous

chemical firing in nerve (Brink and Bronk, 1937) and in causing striking oscillations of potential (Arvanitaki, 1939), the effect of this agent upon tissue rectification was investigated. The procedure used was to soak the tissue first in 0.116 M NaCl and then in 0.116 M NaSCN (Sjöstrand, Brink, and Bronk, 1938). No effect upon rectification was observed, however, with low calcium. Neither do excess calcium (0.083 M) nor barium chloride (0.083 M)

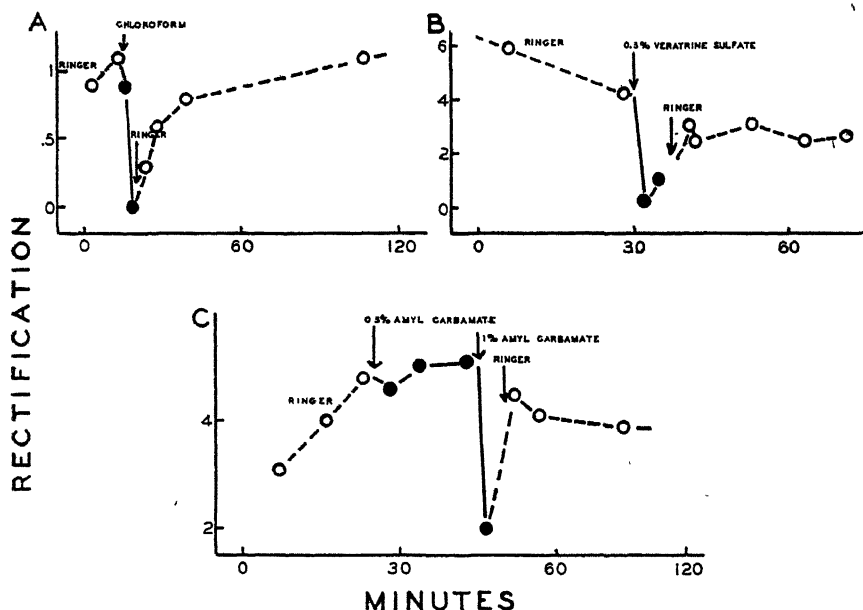


FIG. 4. Effect of narcotics upon rectification in sciatic nerve (A) and sartorius muscle (B and C) of *Rana pipiens*. Rectification in arbitrary units directly proportional to D. C. component of the current passing through the tissue *vs.* time in minutes. A decrease in resting potential accompanied every decrease in rectification. Both rectification and resting potential changes are partially or completely reversible. (Chloroform was applied as a saturated solution in Ringer.)

affect tissue rectification. In a previous paper, it has been shown that even where the alkaline earth ions have in themselves no effect they are capable of neutralizing the depressing effect of potassium upon resting potentials (Guttman, 1940). No similar neutralizing effect of alkali earths upon the depression of rectification by potassium was observed.

Only one experiment was done to investigate the effect of acetylcholine upon tissue rectification. After soaking frog sartorius for  $\frac{1}{2}$  hour in freshly made up 1-5000 eserine sulfate in Ringer's solution (at pH 7.2), 100 gamma per cc. acetylcholine had no effect upon rectification. Of course, no conclu-

sions can be drawn from an isolated trial of this kind, but the result is mentioned inasmuch as the subject is of interest to many.

#### DISCUSSION

*Rectification Effect Located in Membrane.*—The fact that the potassium ion decreases rectification in the tissue may be of assistance in determining where in the cell rectification occurs. One would not expect the potassium ion to be able to affect the rectification of (1) the Ringer's solution, (2) the connective tissue, or (3) the protoplasm of the nerve or muscle cell interiors, so as to alter the degree of rectification exhibited by them, supposing for the moment that these media were capable of rectification. We do know, on the other hand, that potassium profoundly influences the membrane structure of nerve and muscle cells. Since the data here presented demonstrate that potassium markedly affects the degree of rectification exhibited by nerve and muscle cells, there is some indication that the seat of rectification is in the cell membrane. (Evidence for the assumption that the cell interiors and the external media act merely as electrolytes may be obtained from the transverse resting impedance data of Cole.) These data may thus be considered as experimental verification of Cole's suggestion that selective ion permeability of a membrane may be expected to give rise to rectification (Cole, 1941) since they show that when the membrane is externally in contact with ions to which it is more permeable or has its permeability increased by narcotics rectification falls off.

*Mechanism of Rectification.*—The experiments with potassium offer experimental verification of Cole's suggestion (1941) that the mechanism of rectification may probably be explained on the basis of ionic conduction rather than electronic conduction in the cell membrane. They are of especial interest in connection with his more specific supposition that rectification may possibly be explained on the basis of conduction by potassium ions alone. Since the external concentration of potassium is low an inwardly flowing current would decrease the potassium ions in the membrane and decrease its conductivity. An outwardly flowing current would have the opposite effect since the internal concentration of potassium ions is relatively high. Thus rectification in the membrane, *viz.* a change in resistance with change in direction of current flow, may possibly be explained in terms of a change in the concentration of potassium ions in the membrane.

In the potassium experiments described in this paper, rectification was decreased or disappeared when the concentration of potassium ions in the outer medium was increased and the gradient of potassium ions in the membrane thus lessened. This work constitutes experimental verification of a possible explanation for rectification in living tissues.

Such an explanation for rectification is similar to that long ago suggested for resting potentials. Höber (1905) has shown for frog muscle, Osterhout (1931)



for *Nitella*, and Cowan (1934) for crab nerve that bathing cell exteriors with KCl solutions and thus lessening the potassium gradient normally present in the membrane will decrease the potential, and Blinks (1930) showed that increasing external KCl increased the conductivity in *Nitella*. In almost every experiment reported in this paper, resting potentials were observed at the same time as rectification. Invariably, whenever the rectification of the tissue was decreased, the resting potential also declined. This was true not only when the cells were treated with potassium but also when narcotics were used. (That narcotics decrease resting potentials has long been known (Höber, Andersch, Höber, and Nebel, 1939; Guttman, 1940).)

That it was possible to lessen rectification (and resting potentials) by narcotics as well as by increasing the external potassium, may indicate that narcotics increase permeability to all ions.

The author wishes to acknowledge the untiring cooperation of Mr. Charles Sheer, of the RCA Institute and the Department of Neurology, Columbia University, who designed and built the measuring circuit, and of Mr. Marcel Thienpont, now of the U. S. Navy, who assisted him. The author is also deeply grateful to Professor Martin Meyer of Brooklyn College for making research space available and to the Departments of Hygiene, Physics, Chemistry, and Biology of Brooklyn College for the loan of apparatus and materials. The assistance of Professor Kenneth S. Cole, now at the Metallurgical Laboratory, University of Chicago, during the preliminary experiments performed at Woods Hole is gratefully acknowledged.

#### SUMMARY

Electrical rectification was demonstrated in whole sartorius muscle and sciatic nerve of *Rana pipiens* and also in the single giant nerve fiber of the northern squid, *Ommastrephes illecebrosus*. It is probably a property of the plasma membrane.

Rectification decreases reversibly under the influence of increased concentrations of the potassium ion and with chloroform, veratrine sulfate and isoamyl carbamate. No effect was found with lack of calcium, excess calcium, or barium chloride.

Decrease in rectification is invariably accompanied by simultaneous decrease in resting potential.

A proposed explanation of the mechanism of rectification is discussed. Rectification in a living membrane, *viz.* a change in resistance with change in direction of current flow, may possibly be explained in terms of a change in the concentration of potassium ions in the membrane.

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# THE PENETRATION OF AMMONIA INTO FROG MUSCLE

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These experiments were undertaken in order to discover to what extent ammonium behaves like potassium in skeletal muscle, and a preliminary report has already been made of the results obtained (Fenn, Haege, and Sheridan, 1942).

The general plan was simply to replace a part of the NaCl in Ringer's solution by  $\text{NH}_4\text{Cl}$  and to compare the composition of a muscle after immersion in this solution with the composition of its mate immersed under similar conditions in Ringer's solution. A few experiments were tried with other frog tissues, especially liver. The results have shown some tendency for the  $\text{NH}_3$  to become concentrated in the muscles like K, but the inside-to-outside concentration ratios are very different for the two cations.

The ammonia enters mostly in exchange for K and Na. It does not take chloride in with it because there is no evidence of swelling of the muscle. This confirms the idea that the muscle membrane is permeable to cations but not to anions.

## Methods

We have used mostly the small hind leg muscles of *Rana pipiens* including the sartorius, semitendinosus, ileofibularis, peroneus, and tibialis. Sometimes larger muscles such as the gastrocnemius have been used, but these have usually been cut longitudinally into 2 or 3 smaller pieces. About 0.7 to 1.0 gm. of muscle was soaked in 1.5 to 2.5 ml. of solution in small weighing bottles with ground glass and greased stoppers and equilibrated with  $\text{O}_2$ . These bottles were then rotated slowly around an axis inclined about  $30^\circ$  from the vertical at  $19^\circ\text{C}$ . or  $5^\circ\text{C}$ . for periods of time varying from 15 minutes to 5 days.

The control solution contained 0.65 per cent NaCl, 0.01 per cent KCl, 0.02 per cent  $\text{CaCl}_2$ , and  $\frac{M}{150}$  phosphate buffers at pH 7.0. This was compared with the same solution with  $\frac{1}{5}$  of the NaCl replaced by an equivalent amount of  $\text{NH}_4\text{Cl}$ . The latter solution contained 22.8 m. eq. of  $\text{NH}_3$  and had the same pH as the control solution within 0.1 of a pH unit. The two solutions will be referred to as the R and the N solution respectively.

Potassium was determined by a somewhat modified Shohl and Bennett

method (Fenn *et al.*, 1938).  $\text{NH}_3$  was steam-distilled *in vacuo* by a simplification of the method recommended by Parnas and Heller (1924). The ammonia was caught in dilute  $\text{H}_2\text{SO}_4$  (or boric acid) and titrated. By properly adjusting the pressure in the condenser to equal the vapor pressure of the solution, it was possible to run the distillation without losing any bubbles through the  $\text{H}_2\text{SO}_4$  and without heating the solution.

The  $\text{NH}_3$  content of muscles was determined in the same way after snipping up the muscles with scissors in a chilled alkaline borate solution and then transferring them to the distillation flask. Small muscles can be dropped into the borate solution whole. In some experiments the muscles were ground up in sand in the borate solution, as recommended by Parnas and Mozolowski (1927), but this permitted a loss of 10 per cent of the  $\text{NH}_3$ . In some of the earlier experiments, we made the mistake of adding strong alkali to the distillation flask, thereby forming some ammonia from the amide nitrogen present (*cf.* Schmidt, 1938). Since, however, this error was equal in both the control and the experimental muscle, the difference in  $\text{NH}_3$  content was presumably unaffected and, indeed, the results obtained by this method (which will not be reported in detail because of this error) were nevertheless in good agreement with figures obtained by the correct procedure. The amount of  $\text{NH}_3$  formed by the strong alkali amounted on the average to 8 m. eq. per kilo. of muscle.

### 1. Changes in the Weight of Muscles and Liver during Immersion

Weight changes in all our experiments are summarized in Table I. For purposes of comparison, the muscles are divided into four groups of more or less equal size. In each of these groups, the average weight of the muscles before and after immersion is given, and, in the last two columns, the percentage change in weight. The most essential point is that the presence of  $\text{NH}_4\text{Cl}$  in the solution does not alter significantly the movements of water. If anything, the muscles in the N solution lose more water or gain less than their mates in the R solution. It is evident, therefore, that the muscles are not like erythrocytes which swell in  $\text{NH}_4\text{Cl}$  because both anion and cation can penetrate, thus increasing the osmotic pressure inside (Jacobs, 1927). As a tentative conclusion, it may be said, therefore, that the muscle is impermeable to chloride. The same conclusion applies to liver, although there is a somewhat greater loss of water in the R than in the N solution. It is interesting to note that the small muscles, each weighing 100 or 200 mg., lost less weight than the larger muscles, weighing more nearly 1 gm. In series 4, the muscles were immersed in 100 ml. of solution instead of 1.5 to 2.5 ml., and the immersion was continued for 1 to 5 days rather than 5 hours as in the other series. In this greater interval, or perhaps because of the larger volume of solution, there was some swelling rather than shrinking of the muscles.

From these results, it is evident that the muscles were not seriously injured by the substitution of part of the Na by  $\text{NH}_4$ . They did not swell or go into rigor. The rheobase excitability was measured in many cases. In ten cases, the average rheobase voltage was 3 times as high in the muscle soaked in  $\text{NH}_4\text{Cl}$  solution as in the control. In spite of the  $\text{NH}_4\text{Cl}$ , however, these muscles did contract, although the threshold was somewhat higher. This confirms a loss of excitability previously reported in preliminary experiments (Fenn and Cobb, 1933).

TABLE I

*Average Weight of Muscles and Liver before and after Immersion in Ringer's (R) and  $\text{NH}_4\text{Cl}$ -Ringer's (N) Solution*

Series	Tissue	No. of muscles	Time	R		N		Per cent change	
				Before	After	Before	After	R	N
			hrs.	mg.	mg.	mg.	mg.		
1	Large muscles	20	5	1102	1086	1100	1073	-1.5	-2.5
2	Large muscles	21	5	1323	1290	1289	1261	-2.5	-2.2
3	Small muscles	13	5	846	840	852	846	-0.71	-0.70
4	1-5 day muscles	16	24-120	601	635	621	643	+5.7	+3.5
5	Liver	13	5	667	622	681	661	-6.8	-2.9

In series 1 and 2, the weights refer to single muscles, while, in series 3, the weights given are the sums of the weights of 4 or 5 small muscles.

## 2. The Volume of Distribution of $\text{NH}_3$ in Muscle and Liver

The volume of distribution is defined as that volume in which the  $\text{NH}_3$  (as  $\text{NH}_4^+$  or otherwise) would have to be distributed inside the tissue in a concentration equal to that in the ambient solution in order to account for all the  $\text{NH}_3$  found in the tissue. When the tissue is immersed in a small volume of solution containing a known amount of  $\text{NH}_4\text{Cl}$ , the final distribution of this salt between the muscle and the solution is fully determined if the amount present in either the solution or the muscle is known by analysis. Thus, if the muscle is analyzed, the amount in the solution can be obtained by difference, and *vice versa*. In some experiments, we analyzed the solution and, in others, the muscle, and, in still others, we analyzed both tissues and solutions. A still simpler method which we followed in some cases was to immerse the muscles in a volume of solution so large that it did not change in concentration during the immersion. We usually used a smaller volume of solution, however, because we were interested in analyzing the solution for potassium escaping from the muscle, and we wished to make the change in concentration as large as possible.

The volume of distribution is calculated as follows. Let  $V$  equal the initial volume of the solution in milliliters and  $m$  the initial wet weight of the muscle

in grams. The initial concentration of the  $\text{NH}_4\text{Cl}$  in m. eq. per ml. is  $C_0$ , and the concentrations after immersion in the R and N solutions are  $C_r$  and  $C_n$ . The initial concentration of  $\text{NH}_4\text{Cl}$  in the control solution at the start is zero, but some  $\text{NH}_3$  diffuses out of the control muscle during the immersion so that  $C_r$  has a definite value. Since the two muscles are nearly equal in weight, it is assumed that the preformed  $\text{NH}_3$  in the experimental muscle will raise the concentration in the N solution to the same extent. If the two muscles differ appreciably in weight, a correction is made to take account of that fact. That fraction of the concentration of  $\text{NH}_3$  in the N solution after soaking which is due to added  $\text{NH}_3$  is therefore  $C_n - C_r$ . This concentration is less than  $C_0$  because some  $\text{NH}_3$  has diffused into some part of the muscle. The calculation is somewhat complicated by the change in weight of the muscle during immersion, but these changes are never very large so that a somewhat simplified calculation is justified. To meet this difficulty, it is assumed that if any water entered or left the muscle during immersion, that water will contain  $\text{NH}_3$  in a concentration equal to  $C_n$ , and the calculation is carried out as if that amount of water were returned to its original position with its contained  $\text{NH}_3$ . The value will, therefore, be unchanged by immersion, and we may write

$$\frac{C_0}{C_n - C_r} = \frac{V + x}{V} \quad (1)$$

where  $x$  is the volume of muscle substance in milliliters in which  $\text{NH}_3$  has diffused in a concentration equal to that in the solution. The volume of distribution in per cent is then given by the equation

$$\frac{100x}{m} = \frac{100V}{m} \left( \frac{C_0}{C_n - C_r} - 1 \right) \quad (2)$$

The volume of distribution can also be calculated from the concentrations of  $\text{NH}_3$  found in the muscles after the period of immersion. Let these concentrations be  $c_r$  and  $c_n$ . Then the concentration in the muscle due to the added  $\text{NH}_3$  will be  $c_n - c_r$ , and the concentration left in the solution will be  $(VC_0 - m(c_n - c_r))/V$ . The volume of distribution will then be given by the equation

$$\frac{100(C_n - C_r)V}{VC_0 - m(c_n - c_r)} = \text{volume of distribution} \quad (3)$$

In this case,  $c_n$  and  $c_r$  were calculated per gram of initial weight of the muscles, so that if the muscles gained weight during the immersion, the calculated volume of distribution will be too large, and *vice versa*.

The results of a large number of experiments calculated by these two equations are summarized in Table II. The protocols of the individual experiments are not given chiefly because, with the exception of series 5, all the analyses

were made by the addition of excessive amounts of alkali to the distillation flask, so that there was some formation of  $\text{NH}_3$  from amide nitrogen. It does not appear, however, that this error was very large when the solutions were being analyzed, nor does it appear that the values of  $C_n - C_r$  or  $c_n - c_r$  were changed by the addition of an equal amount to both muscles or both solutions.

In series 1, the muscles were soaked in Ringer's solution overnight in the cold room before use. They were then weighed and immersed in the R and N solutions for 3 to 5 hours at  $19^\circ\text{C}$ . The average of 22 experiments gives a volume of distribution of 112 per cent. Series 2 was similar except that the muscles were removed from the solutions at the end of the experiments, blotted,

TABLE II  
*Volume of Distribution of  $\text{NH}_3$  in Muscle and Liver*

Series	Tissues	No. of experiments	Average weight of muscles	Average volume of solution	$\text{NH}_3$ in solution mM per liter		Average volume of distribution	P.E. of mean
					R	N		
			gm.	ml.			per cent	
1	Muscle	22	1.24	2.07	1.41	15.36	112	3.4
2	Muscle	18	1.30	2.28	2.12	16.07	111	3.8
3	Liver	8	0.736	1.88	3.65	16.25	216	19.3
4	Liver	5	0.592	2.0	3.09	17.4	208	26
2	Muscle	18	1.30	2.28	9.87*	23.25*	88	3.9
4	Liver	5	0.592	2.0	12.7*	28.6*	80	21
5	Muscle	7	0.813	100	0.87*	15.75*	67	2.2

The weights given are the initial weights of the muscles in the N solutions. Concentrations refer to analyses made after immersion of the tissues. Concentrations in the tissues are calculated per gram initial wet weight of the tissues. Analyses were made both in the tissues and in the solutions. The numbering of the series in this table is not synonymous with that in Table I.

\*  $\text{NH}_3$  in tissue in millimols per kilo.

weighed, and analyzed for  $\text{NH}_3$ . The solution analyses, calculated by equation 1 gave a volume of distribution of 111 per cent, while the tissue analyses calculated by equation 2 gave a lower value of 88 per cent. The exact reason for the discrepancy between these two values is not known, but some of it is due to loss of  $\text{NH}_3$  from the muscles during the process of grinding them in the borate solution. In two control experiments in which  $\text{NH}_4\text{Cl}$  was "ground" in borate in this way, the loss of  $\text{NH}_3$  in the process amounted to 10.7 and 11.0 per cent respectively. In series 5, however, in which the muscle  $\text{NH}_3$  was distilled from alkaline borate only, and a large volume of solution was used a still lower value of 67 per cent was obtained for the volume of distribution. This low value may perhaps be accounted for by the fact that in five of the seven experiments of series 5, the muscles were immersed at  $5^\circ\text{C}$ . instead of  $19^\circ\text{C}$ .



Series 3 and 4 of Table II represent similar experiments with pieces of liver in thin slices. In this case, the volume of distribution was 208 per cent and 216 per cent by solution analysis, and only 80 per cent by tissue analysis. This means that more  $\text{NH}_3$  disappeared from the solution than could be accounted for by the  $\text{NH}_3$  found in the tissues. This difference undoubtedly is real and is due to the formation of urea in the liver (Krebs and Henseleit, 1932). This was verified in one experiment by direct analysis of the liver for urea. Similar analyses were made in six of the muscle experiments. In these experiments, the R solutions contained urea after immersion in concentrations 1.7, 2.1, 3.7, 4.7, and 3.4 m. eq. per kilo respectively, while the corresponding concentrations in the N solutions were 0, 3.3, 2.2, 1.3, 3.5, and 4.6 m. eq. per kilo. On the

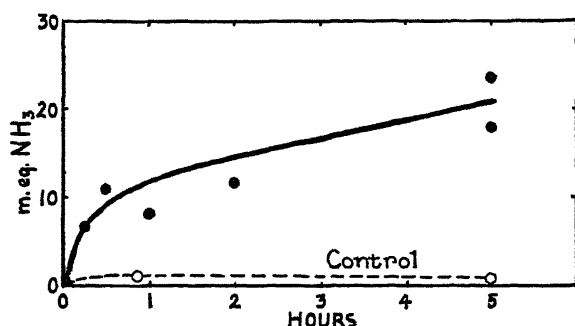


FIG. 1. Ammonium contents of muscles after immersion for various lengths of time in 100 ml. samples of R and N solutions at  $5^{\circ}\text{C}$ . and equilibration with pure oxygen and continuous rotation to insure gentle agitation. All the points marked by circles represent muscles obtained from the same frog, each sample weighing 600 to 800 mg.

average, therefore, the concentrations were 3.1 and 2.5 m. eq. per kilo respectively in the R and in the N solutions, and this difference is not significant. This confirms the finding of Krebs and Henseleit that urea is not manufactured by muscles from  $\text{NH}_3$  and indicates that low values of the volume of distribution obtained by analysis of muscle for  $\text{NH}_3$  cannot be explained by urea formation.

### 3. Time Course of the Penetration of $\text{NH}_3$

The previous experiments were carried out mostly after about 5 hours of immersion. At this time, the volume of distribution is about 100 per cent. Using the improved method of distilling from borate only, an investigation was carried out of the amount of  $\text{NH}_3$  taken up at shorter and longer times. We first studied shorter times, and the results of these experiments are shown in Fig. 1. Ordinates represent mM of  $\text{NH}_3$  per kilo of muscle. The  $\text{NH}_3$  in the control muscle is uniformly about 1 mM per kilo and does not increase with

time. In the experimental muscle, however, exposed to a solution containing  $\text{NH}_3$  in a concentration of 22.8 mM per kilo, the concentration increases in about 5 minutes to about 17 mM per kilo and subsequently increases more slowly and linearly until it reaches a value about equal to that in the solution at 5 hours. This finding confirms, therefore, the volume of distribution of 100 per cent found in the earlier, more numerous but less precise experiments. In interpretation of the curve of Fig. 1, it might be supposed that the initial rapid increase in the first 15 minutes represents the saturation of the extracellular space, while the subsequent slower rise is due to penetration of the muscle fibers themselves.

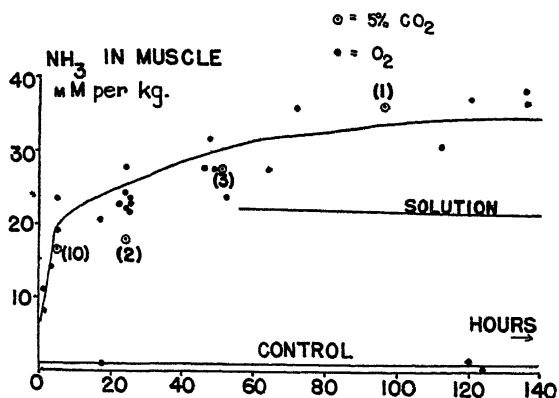


FIG. 2. Similar to Fig. 1, but extending over longer periods of immersion. Points marked by circles were obtained from muscles equilibrated with 5 per cent  $\text{CO}_2$  and 95 per cent  $\text{O}_2$ . Figures on the graph represent the number of points which were averaged together to obtain the value plotted. The final concentration attained after 5 days is greater than the concentration in the solution represented by a horizontal line.

In Fig. 2 are plotted the results of other experiments in which muscles were immersed in  $\text{NH}_4\text{Cl}$  Ringer's solution for several days. This shows that the 100 per cent volume of distribution found at 5 hours did not represent a true equilibrium value because the  $\text{NH}_3$  concentration continues to increase slowly until the volume of distribution reaches a maximum value of about 150 per cent at the end of 4 or 5 days. During this time, there is evidently some accumulation of  $\text{NH}_3$  against the concentration gradient. Similar results were obtained in *Valonia* by Cooper and Osterhout in 1930.

#### 4. The Effect of $\text{NH}_4\text{Cl}$ on the Loss of Potassium from Tissues

In a previous preliminary investigation (Fenn and Cobb, 1933) it was found that  $\text{NH}_4\text{Cl}$  accelerated the loss of K from frog muscle, but more quantitative

data were desirable. For this purpose, the tissues were accordingly suspended in small volumes of solutions, samples of which were withdrawn at intervals for potassium analyses. At the end of the experiment, the muscles themselves were analyzed for potassium. By adding to the final content the amounts of potassium found in the solution, the original amount present could be calculated. In this way, the data of Fig. 3 were obtained. The potassium content of the muscles immersed in the  $\text{NH}_4\text{Cl}$ -Ringer solution fell more rapidly than that of the control muscles in Ringer's solution. This was especially true in the 1st hour. At the end of the experiment, the control muscle contained 13.6 m. eq. per kilo more K than the experimental muscle, while the  $\text{NH}_3$  content calcu-

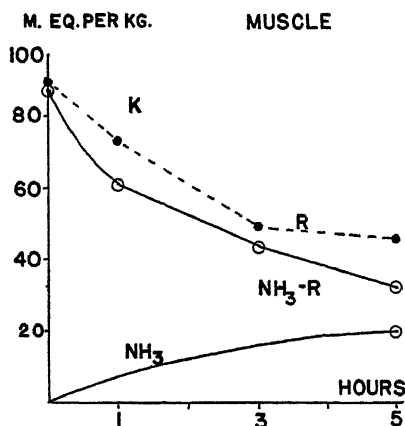


FIG. 3. The loss of potassium from muscles immersed in Ringer's solution is greater if the solution contains some  $\text{NH}_4\text{Cl}$ . The original solutions contained 1.49 m. eq. of K per liter. Samples (0.437 ml.) removed at 1 hour contained 3.47 and 3.84, and those removed at 3 hours contained 5.93 and 6.54 m. eq. of K per liter in the R and N solutions respectively. The final concentrations after 5 hours and other data are given in Table III (last striated muscle experiment).

lated for a volume of distribution of 100 per cent would be 19.8 m. eq. per kilo. (The  $\text{NH}_3$  contained in 2 ml. of solution at an initial concentration of 22.8 m. eq. per kilo diffuses into a muscle weighing 0.222 gm. until the concentrations in solution and muscle are equal. Some similar experiments for other tissues are plotted in Fig. 4.) In every case, it is apparent that the loss of potassium was greater in the tissue immersed in the solution containing  $\text{NH}_4\text{Cl}$ . No actual analyses for  $\text{NH}_3$  were made in any of these particular experiments, so that the graphs for  $\text{NH}_3$  must be regarded as diagrammatic. They are, however, in accord with  $\text{NH}_3$  analyses made in other similar experiments already described.

A summary of the data from experiments of this type is given in Table III.

The amounts of potassium which diffuse from the muscle into the solution because of the  $\text{NH}_4\text{Cl}$  can be calculated from the potassium contents of both the tissues and the solutions. The error of both these determinations is large because the errors of several potassium analyses are included. On the average, however, it can be seen that 9.75 m. eq. per kilo were lost from the tissues (assuming equal concentrations in the matched muscles at the start) while 9.25 m. eq. per kilo were found in the solutions. Large discrepancies were

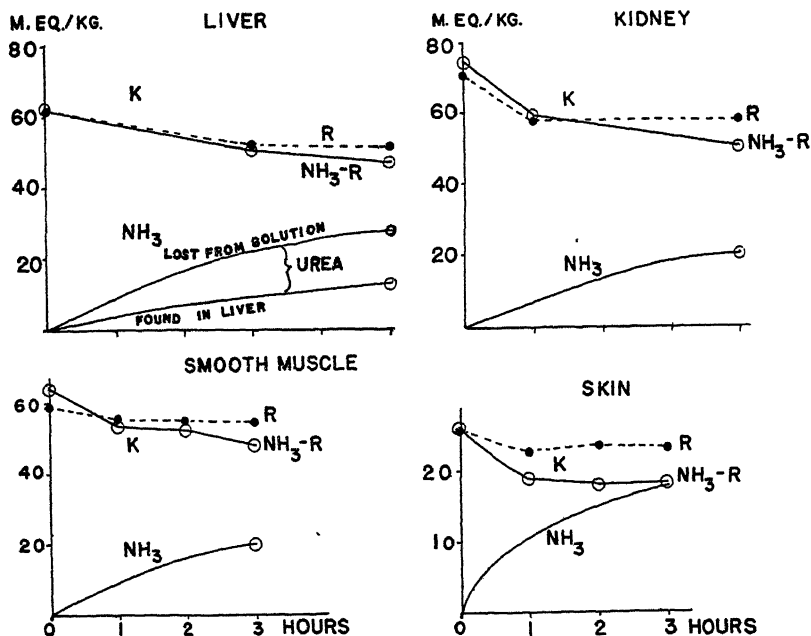


FIG. 4. Loss of potassium from tissues immersed in Ringer's solution with and without  $\text{NH}_4\text{Cl}$  substitution. Data concerning these experiments are given in Table III (see the second liver experiment). Further description in text.

found in some individual experiments. For comparison with this figure, there was a calculated amount of  $\text{NH}_3$  diffusing in the opposite direction equal to 17.3 m. eq. per kilo. The  $\text{NH}_3$  might, therefore, enter by exchange with other cations if potassium were not the sole cation involved. In *Valonia* also the penetration of  $\text{NH}_3$  is accompanied by a nearly equivalent loss of K (Cooper and Osterhout, 1930).

The relation between the amount of K lost and the amount of ammonia gained is shown also in the data of Table IV. In these experiments, the tissue was analyzed directly for K while the solutions were analyzed for  $\text{NH}_3$ . The change in K reported in the table represents the difference between the K

TABLE III

*Loss of K from Tissues after Soaking in Ringer's Solution with (N) and without (R)  $\text{NH}_4\text{Cl}$*

The weight of the piece of tissue suspended in the N solution is given in column 4 and the volume of the solution is contained in column 3. The weight of the control tissue was very similar in all cases. The final weights of both tissues are given in columns 5 and 6 in per cent of their respective initial weights. With a special pipette calibrated to contain 0.437 cc., samples were removed at intervals of a few hours for analysis. At the end of the experiment, the tissues were analyzed, and the results are shown in columns 7 and 8, the difference being given in column 9. At the same time the remaining solutions were analyzed, the results being listed in columns 10 and 11. Column 12 is not the difference between columns 10 and 11, but represents the total amount of extra K found in the N solutions, including the amounts removed in intermediate sampling during the experiment. This amount is represented as a concentration by dividing it by the initial volumes given in column 3. Column 13 equals (column 12)  $\times$  (column 3) and divided by (column 4). Column 13 represents the amount of K lost from the tissue as calculated from the extra amount found in the solution (column 12) and should be equal to column 9. Column 14 equals 22 m. eq. per kilo  $\times$  (column 3) and divided by (column 3 plus column 4). In the case of liver, the weight is multiplied by 2.11 for this formula to allow for a volume of distribution of 211 per cent.

1	2	3	4	5	6	7	8	9	10	11	12	13	14
Tissue	Time	Volume of solution	Initial weight	Final weight in per cent of initial		K in tissue m.eq. per kg. initial weight			K in solution Final concentration in m.eq. per l.			K m.eq. per kg. of Tissue	$\text{NH}_3$ m.eq. per kg. of Tissue
				R	N	R	N	$\Delta\text{K}$	R	N	$\Delta\text{K}$		
	hrs.	ml.	gm.										
Muscle	5.0	1.5	0.336	92.1	95.8	76.0	56.3	19.7	4.5	6.3	1.8	8.0	18.0
Muscle	5.0	1.5	0.198	97.2	101.4	65.8	51.8	14.0	4.56	6.82	2.26	17.1	19.4
Muscle	5.0	2.0	0.298	117.4	132.2	67.3	61.2	6.1	3.13	5.02	1.89	12.7	19.1
Muscle	5.0	2.0	0.222	119	117	45.8	32.2	13.6	6.61	8.75	1.36	12.2	19.8
Liver	5.0	1.0	0.752	110	108.4	56.9	45.6	11.3	8.6	9.7	1.1	1.5	8.5
Liver	5.0	2.0	0.638	119	124	51.1	46.6	4.5	4.7	7.1	1.58	4.95	13.2
Liver	3.0	2.0	0.239			70.3	61.2	9.1	1.77	3.38	1.1	9.3	17.6
Kidney	2.0	1.5	0.197			58.5	51.0	7.5	2.12	3.13	0.75	11.5	20.6
Smooth muscle	3.0	1.5	0.266			54.5	47.8	6.7	2.32	4.37	1.68	9.5	18.7
Skin	3.0	1.5	0.355			23.2	18.2	5.0	2.03	3.44	1.52	6.4	17.8
Average—for skeletal muscle . . . . .									13.4			12.4	18.7
Grand average . . . . .									9.75			9.25	17.3

TABLE IV

*Potassium Loss and Ammonium Gain of Muscles in 5 Hours*

Control K, m.eq. per kg. . . .	42.1	42.5	49.5	53.4	73.8	53.0	48.9	38.2	53.3	60.7	48.0	62.0	60.9	Average
$\Delta\text{K}$ , m.eq. per kg.	7.3	1.9	-10.2	-9.0	-19.9	-3.9	-8.9	2.2	1.4	-13.4	0.9	-8.1	-17.7	-6.0
$\Delta\text{NH}_3$ , m.eq. per kg. . . . .	22.8*	22.8*	22.8*	22.8*	19.4	15.4	15.5	14.0	14.8	15.4	19.7	13.7	20.7	16.5

A minus sign means a loss from the muscle. No sign means a gain.

\* Calculated, assuming a volume of distribution = 100 per cent.

contents of the control and experimental muscles at the end of the experiment. In ten of these thirteen experiments, the potassium loss was greater in the presence of  $\text{NH}_4\text{Cl}$ . The other three cases were doubtless due to experimental error. On the average, the gain in  $\text{NH}_3$ , averaging 16.5 m. eq. per kilo, was considerably greater than the loss of K which averaged only 6 m. eq. per kilo. It should be noted, however, that in these experiments the muscles were soaked overnight in Ringer's solution before the experiment. During this time, a considerable amount of K was lost. When the experiment was performed with-

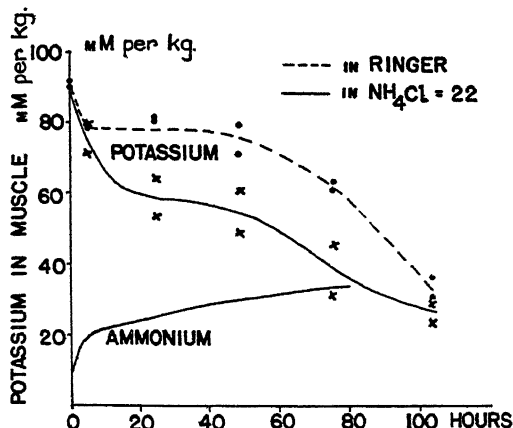


FIG. 5. Loss of potassium from muscles immersed in Ringer's solution with and without substituted  $\text{NH}_4\text{Cl}$ . The curve for penetration of  $\text{NH}_3$  from Fig. 2 is included for comparison. These muscles were immersed for varying periods of time in large volumes of solutions (100 cc.) at about  $5^\circ\text{C}$ . In addition to these experiments, four additional pairs of muscles were analyzed after immersion under similar conditions for 24 and 48 hours. In these eight pairs of analyses, the mean difference in potassium content was 19.2 m. eq. per kilo. Six other similar muscles were analyzed for  $\text{NH}_3$  after immersion for 24 (four muscles) and 48 (two muscles) hours, and were found to have an average  $\text{NH}_3$  content of 24.2 m. eq. per kilo.

out this preliminary soaking, as in the experiments of Table III, the loss of K due to the  $\text{NH}_3$  was 12.4 to 13.4 as shown instead of 6 m. eq. per kilo. This value is more nearly equal to the  $\text{NH}_3$  gain. It is possible that during the soaking overnight some of the muscle K exchanged for Na, and, later, some of this Na exchanged in turn with  $\text{NH}_4$ . It seems likely, therefore, that all the  $\text{NH}_3$  which enters does so in exchange for an equivalent amount of some other cation.

Other similar experiments were run over longer periods of time in order to see whether, at a point of more complete equilibrium, the equality between K loss and  $\text{NH}_3$  gain might be more striking. The results of these experiments are to be seen in Fig. 5, where the potassium contents of the control and ex-

perimental muscles are plotted against time. The concentration of  $\text{NH}_3$  in the muscle, as given in Fig. 2, is also included for comparison. According to the chart, it requires about 20 hours for the difference in potassium content of the two muscles to reach its maximum. At this point, the experimental muscle in  $\text{NH}_4\text{Cl}$  has lost about 20 m. eq. per kilo more K than the control muscle and this difference is about equal to the concentration of the  $\text{NH}_4\text{Cl}$  in the Ringer's solution which was 22 m. eq. per kilo. At 20 hours, however, the  $\text{NH}_3$  content is already 24 m. eq. per kilo or slightly greater than the concentration in the solution, and this concentration continues to increase to about 35 at the end of 140 hours. While, therefore, there is a close relationship between the loss of K and the gain in  $\text{NH}_3$ , the two quantities are never exactly equal, and it seems likely that other cations are involved in the equilibrium.

The question of the reversibility of the loss of potassium caused by the penetration of  $\text{NH}_3$  is an important one for which we have no answer. In one experiment, we endeavored to obtain information on the point by exposing matched muscles to the  $\text{NH}_4\text{Cl}$ -Ringer's solution overnight. In the morning, one muscle was analyzed for potassium, while the other was replaced in Ringer's solution without  $\text{NH}_3$ . When the second muscle was analyzed some hours later, its potassium content was still lower than that of its mate. The result shows only that, in Ringer's solution when  $\text{NH}_3$  diffused out of the muscle, the potassium was not replenished at a rate which was fast enough to exceed the continuous loss due to disintegration of the muscle. These exchanges are apparently so slow in muscle that reversibility is difficult to demonstrate. According to Conway, O'Brien, and Boyle (1941) potassium of yeast can be completely replaced by  $\text{NH}_3$ , and this exchange can be reversed, but even in this small cell the exchange is not rapid.

### *5. Experiments with Radioactive Potassium*

Evidence already presented has indicated that the  $\text{NH}_3$  enters partly at least in exchange for K, and it has been shown that the loss of K is greater in the muscle immersed in the  $\text{NH}_3$  solution. This conclusion is confirmed by another experiment in which artificially radioactive potassium ( $\text{K}^{42}$ ) was used. A solution of radioactive K was injected into the dorsal lymph sac of a frog the day before the experiment. After this period of time, it can be assumed that  $\text{K}^{42}$  has completely mixed with  $\text{K}^{39}$  throughout the muscles of the body (Noonan, Fenn, and Haege, 1941). Muscles were then dissected as usual and were immersed in R and N solutions according to the usual technique. At intervals samples of the solutions were transferred to the cup surrounding the ionization chamber of a Geiger-Müller counter for a count of the  $\beta$  rays. The radioactivity of the solution presumably depends under these conditions (1) on the net amount of radioactive K lost from the muscles and (2) on the amount exchanged between the  $\text{K}^{42}$  in the muscle and the  $\text{K}^{39}$  of the solution. The

latter factor would presumably be the same for both muscles, but the net loss would be greater in the  $\text{NH}_3$  muscle because of exchange of K for  $\text{NH}_4$ . The graphs of Fig. 6 are in accord with this expectation for the  $\beta$  ray count was always higher in the presence of  $\text{NH}_4\text{Cl}$ .

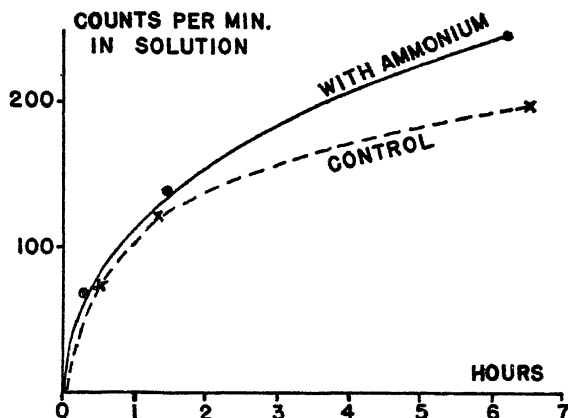


FIG. 6. Matched muscles taken from frogs previously injected with radioactive  $\text{K}^{42}$  were immersed in R and N solutions. The results show that K escapes more rapidly when it can exchange with  $\text{NH}_4^+$  and the  $\beta$ -ray count is always higher in the Ringer +  $\text{NH}_4\text{Cl}$  solution.

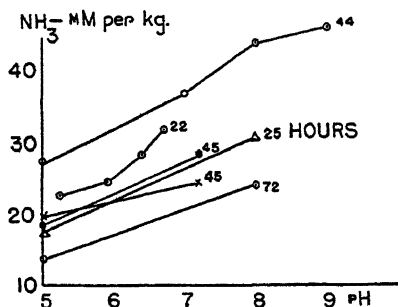


FIG. 7.  $\text{NH}_3$  content of muscles immersed in  $\text{NH}_4\text{Cl}$ -Ringer's solution at varying pH. Alkalinity favors accumulation of  $\text{NH}_3$  in the muscle. Figures indicate the number of hours of immersion. Each graph represents one experiment on muscles of one frog. The solution contained 22 m. eq. of  $\text{NH}_4\text{Cl}$  per l.

#### 6. The Effect of pH

The effect of varying the pH of the solution is shown in Fig. 7. Each point represents the difference in  $\text{NH}_3$  content between the control and the experimental muscle after immersion for varying lengths of time in the R and N



solutions. The duration of the immersion varied from 22 to 72 hours as indicated in the figure. Each graph represents an experiment with muscles from a single frog, each point being the result of analyses of a single pair of those muscles. In all cases, the  $\text{NH}_3$  content is higher in the more alkaline solution. This is in accord with the theory of Osterhout (1930), according to which the  $\text{NH}_3$  penetrates as undissociated  $\text{NH}_4\text{OH}$ , the concentration of which is proportional to the product of the concentrations of  $\text{OH}^-$  and  $\text{NH}_4^+$  ions. It is also in accord with the idea that  $\text{NH}_4^+$  enters in exchange for  $\text{H}^+$  because the  $\text{H}^+$  would come out more readily into a more alkaline solution. Similar results were reported by Cooper and Osterhout in 1930, for *Valonia*.

In this connection, it may be recalled that the loss of K from muscles immersed in Ringer's solution is greater in the more acid solutions (Fenn and Cobb, 1933). In the presence of  $\text{NH}_4\text{Cl}$ , however, this result might be expected to be changed because, in the alkaline solutions, the  $\text{NH}_3$  penetrated better and released K or exchanged with K. To the extent, then, that the loss of K depends upon the penetration of  $\text{NH}_3$ , its loss will be increased by alkalinity in the solution. In one experiment, two sets of matched muscles were put into  $\text{NH}_4\text{Cl}$ -Ringer's solution, one set of muscles at pH 6.0 and the other at pH 7.7. Both the muscles and the solutions were analyzed for K after the period of immersion. The results showed no consistent difference between the amounts of K lost by the muscles in the two solutions. In the absence of  $\text{NH}_4\text{Cl}$ , the loss would undoubtedly have been greater at pH = 6.0.

### 7. Miscellaneous Factors

A number of other variations in the experiment were also tried, and the results may be conveniently summarized in Table V. The equilibration of the solutions with a gas mixture containing 95 per cent oxygen and 5 per cent  $\text{CO}_2$ , instead of pure oxygen, decreased slightly the penetration of  $\text{NH}_3$ , and this is in accord with the pH effect shown in Fig. 6. Equilibration with pure nitrogen instead of pure oxygen also decreased the penetration of  $\text{NH}_3$ , probably because of the increased acidity due to the lactic acid formed anaerobically. The degree of stirring of the muscles and the solutions was found to have some importance, for, if the muscles were left without any agitation, the penetration decreased from 30.8 to 25.3 mm per kilo. This result was duplicated in several experiments. An increase in the concentration of Ca, Mg, K, or Na in the Ringer solution also caused a decrease in the amount of  $\text{NH}_3$  which accumulated in the muscles in the course of 5 hours. This suggests that  $\text{NH}_4$  could exchange with any other cation. The four experiments in which the Na content was varied from 1.3 to 10.2 (Table Vh) were accomplished by replacing NaCl by isotonic sucrose solution. Both solutions contained the usual amount of  $\text{NH}_4\text{Cl}$  and phosphate buffer. The results are presumably due to the Na rather than to any specific effect of the sucrose.

From Table V, i, it is evident that an increase in the temperature of the

TABLE V

*The Effect of Various Factors on the Penetration of Ammonia into Muscles Immersed in Ringer's Solution Containing 0.022 N  $\text{NH}_4\text{Cl}$*

	Solution		$\text{NH}_3$ in muscle		Time
	A	B	$\frac{A}{m} \times 10^{-3}$	$\frac{B}{m} \times 10^{-3}$	
a.	$\text{O}_2$	5 per cent $\text{CO}_2$	19.3 17.8 23.7	18.0 16.4 19.0	hrs. 23 5 5
b.	$\text{O}_2$	$\text{N}_2$	20.3 34.9	14.0 31.7	24 20
c.	Rest	Stir	25.3	30.8	24
d.	$\text{Ca}=0$	$\text{Ca}=4$	37.3 31.5	28.9 28.2	44 21
e.	$\text{Mg}=0$	$\text{Mg}=4$	29.4 37.0	26.1 35.8	46 47
f.	$\text{K}=1.3$	$\text{K}=21.3$	22.8 27.4	17.2 21.1	22 24
g.	$\text{K}=2$	$\text{K}=8$	14.9 12.6	11.2 11.8	24 24
h.	$\text{Na}=1.3$ plus sucrose	$\text{Na}=10.2$	32.8 29.1 41.1 39.85	26.2 29.0 37.45 38.70	24 27 45 41
i.	$5^\circ\text{C.}$	$23^\circ\text{C.}$	11.3 14.9 19.0 23.9	12.7 17.5 24.8 28.4	1 3 5 18
j.	Small muscles	Large muscles	19.83	18.78	5 to 48
k.	$\text{NH}_3=11.2$	$\text{NH}_3=22.4$	13.0 19.8 19.1	29.0 31.6 36.3	17 41 96
l.	—	$\text{CHCl}_3$	22.0 29.3	21.0 28.7	21 42
m.	—	Boil	23.2	21.0	24

All concentrations are in m. eq. per kg. unless otherwise noted.

Figures in j are averages of eight experiments.

solutions from 5°C. to 23°C. also accelerated the diffusion process and increased the 5 hour concentration of  $\text{NH}_3$  found in the muscles.

The data cannot be considered adequate for the calculation of a diffusion coefficient, but the indications certainly are that the  $Q_{10}$  is small enough to be a diffusion process. A limitation of the process by diffusion is also suggested by the lower concentration found in large muscles compared to small ones. The figures given are the averages of 8 separate determinations, in 6 of which the small muscles contained the most  $\text{NH}_3$ . The average weights of the large and small muscles were respectively 841 and 153 gm. Periods of immersion were 5 hours 1 day, and 2 days in 4, 2, and 2 comparisons respectively. Evidently even the largest muscles are nearly at equilibrium in these times because the difference in the  $\text{NH}_3$  content is small for so large a difference in size of muscle.

In several experiments (Table V, k), matched muscles were immersed in solutions containing 11.2 and 22.4 m. eq. per kilo of  $\text{NH}_4\text{Cl}$  respectively. The  $\text{NH}_4\text{Cl}$  was introduced as usual in exchange for an equivalent amount of  $\text{NaCl}$ . Thus the concentration of  $\text{NH}_3$  was twice as great in the stronger solution, and the concentration of the  $\text{NH}_3$  in the muscles was found to be, on the average, 1.9 times as great. This shows that the amount diffusing is closely proportional to the concentration outside.

Finally, we tried some experiments with dead muscles, killed either by boiling or by chloroform (Table V, l and m). The addition of a drop of chloroform to the flask containing muscles in 0.022N  $\text{NH}_4\text{Cl}$ -Ringer's caused a slight decrease in the amount of  $\text{NH}_3$  taken up, but, in one of the two experiments which ran for 42 hours, the ultimate concentration of the  $\text{NH}_3$  in the chloroformed muscle was, nevertheless, considerably greater than that of the solution, thus indicating that the dead muscle could still exert some concentrating effect on the  $\text{NH}_3$ . The most remarkable feature of this experiment, however, was the result obtained when a similar pair of muscles were analyzed for K at the end of the experiment after immersion in similar solutions. In this case, the chloroformed muscle contained only 60 per cent as much K as its mate in the same  $\text{NH}_3$ -Ringer's, but without chloroform. The ability to hold K was apparently lost without eliminating altogether the ability to concentrate  $\text{NH}_3$ . In another experiment, both muscles were put into  $\text{NH}_3$ -Ringer's, but one of the muscles was heated to 53°C for 5 minutes until it was well contracted. This served to reduce slightly the amount of  $\text{NH}_3$  taken up, and the concentration of the  $\text{NH}_3$  in the dead muscle was 21 mM per kilo of muscle or about 26 mM per kilo of muscle water. This is slightly greater than the concentration in the solution but the difference can probably be attributed to endogenous  $\text{NH}_3$ .

#### *8. Exchange of Sodium and Chloride*

To obtain information concerning the total electrolyte balance in these muscles, some experiments were tried in which the muscles were analyzed

for chloride and for sodium. Chloride was determined by titration with KSCN after complete ashing in  $\text{HNO}_3$ , according to the method of Van Slyke as modi-

TABLE VI

Effect of  $\text{NH}_4\text{Cl}$  on the sodium content of muscles. Muscles were soaked in Ringer's solution (R) or in the same with 0.022N  $\text{NH}_4\text{Cl}$  substituted for an equivalent amount of  $\text{NaCl}$  (N). Immerse in 50 cc. of solution at  $4^\circ\text{C}$ . for 30 hours. The first three sets of muscles were taken from one frog, and the last three from another.

Muscles		Sodium content after immersion (m. eq. per kg. initial weight)	
No. of muscles	Weight of muscles	R	N
	mg.		
2	1077	41.4	35.5
6	628	57.8	53.9
3	872	53.3	49.9
2	1269	44.3	30.0
6	979	69.5	53.0
3	734	57.9	64.8
Average (per kg. initial weight) . . . . .		54.0	47.9
Average (per kg. final weight) . . . . .		52.9	45.0

TABLE VII

Effect of  $\text{NH}_4\text{Cl}$  on the chloride content of muscles. Muscles soaked in Ringer's solution (R) or in the same with 0.022N  $\text{NH}_4\text{Cl}$  substituted for an equivalent amount of  $\text{NaCl}$ . Immerse at  $4^\circ\text{C}$ . for 18 hours.

No. of muscles	Weight of muscles	Chloride after soaking per kgm. initial weight		Initial chloride
		R	N	
	mg.	m. eq.	m. eq.	m. eq. per kg.
1	920	29.2	32.4	9.2
1	519	32.4	34.9	11.2
1	645	43.2	51.7	10.5
4	660	63.4	68.9	11.4
2	509	36.7	38.9	14.6
1	595	48.5	61.4	10.9
Average (per kg. initial weight) . . . . .		42.2	48.0	11.3
Average (per kg. final weight) . . . . .		42.1	46.6	

fied by Manery *et al.* (1938). Sodium was determined gravimetrically after complete dry ashing in a furnace, according to the method of Butler and

Tuthill (1931). The muscles were analyzed after soaking overnight in the cold room. The results show that the chloride content was increased from 42.2 to 48.0 by the  $\text{NH}_3$  (Table VI), while the sodium was decreased from the control value of 54.0 to 47.9 m.eq. per kilo (Table VII). The chloride contents of both muscles are, of course, much increased by soaking as compared to the initial value of 11.3 m. eq. per kilo (obtained by analyzing other similar muscles from another frog which were dissected quickly without any contact with Ringer's solution during the process). The same was true for the sodium contents, but no initial sodium contents were determined in this series of experiments. It is interesting to note, also, that samples which consisted of four to six small muscles such as the sartorius, semitendinosus, ileofibularis, and tibialis took up more of both chloride and sodium than the larger muscles. This is possibly due to a greater relative amount of connective tissue in the smaller muscles. The chloride content, after immersion, is so large that it hardly seems possible to account for it all in the extracellular spaces, and one must suppose that some of it is inside the cells. The sodium analyses suggest that some of the  $\text{NH}_3$  enters in exchange for Na, which entered or otherwise would have entered in combination with the chloride.

#### *9. Effect of $\text{NH}_4\text{Cl}$ on Muscle pH and Bicarbonate Content<sup>1</sup>*

If the ammonia enters the muscle as  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$ , then it might be expected to make the muscle more alkaline inside. If, however, it enters as  $\text{NH}_4$  in exchange for K, then no great change in pH would be expected (Osterhout, 1930). In order to elucidate this point, pairs of muscles were soaked in the R and N solutions as before, and, after soaking, they were transferred to bottles of differential volumeters in an atmosphere of 5 per cent  $\text{CO}_2$  in  $\text{O}_2$  and analyzed for  $\text{HCO}_3$  by tipping citric acid on them from a side arm after temperature equilibrium had been established. The  $\text{CO}_2$  evolved was measured and calculated as volume per cent or milliliters of  $\text{CO}_2$  per 100 gm. of muscle. The muscles were originally soaked at  $5^\circ\text{C}$  or  $19^\circ\text{C}$  for varying periods of time and with constant gentle agitation. In most of the experiments, the muscles were blotted gently when they were removed from the solution, weighed on a torsion balance, and placed on the dry bottom of a flask. In another series of experiments, however, they were immersed in the respirometer bottle in 0.5 cc. of unbuffered Ringer's solution.

The results of these two experiments are shown in Tables VIII and IX. When the muscles were in the dry bottles (Table VIII), the control and experimental muscles contained, on the average, 8.3 and 8.4 volumes per cent of  $\text{CO}_2$  respectively, but, when further exchange with a solution was permitted, the  $\text{CO}_2$  evolved was 15.2 and 24.0 volumes per cent respectively (Table IX).

<sup>1</sup> Experiments of J. B. F.

TABLE VIII

*Effect of  $\text{NH}_4\text{Cl}$  on the  $\text{CO}_2$  Content of Muscles Measured in Dry Respirometers*

Time	Temp.	$\text{CO}_2$ content	
		R	N
<i>hrs.</i>	$^{\circ}\text{C.}$	<i>cc./100 gm.</i>	<i>cc./100 gm.</i>
4	5?	8.9	16.5
4	5?	22.7	20.0
4	5?	21.3	19.9
5	5	1.5	3.3
5	5	8.3	7.6
5	5	8.8	8.5
18.5	5	4.0	5.2
18.5	5	2.6	1.8
24	5	6.2	6.1
24	5	5.8	6.1
43	5	4.6	4.6
1.2	19	7.2	11.4
1.1	19	11.9	11.5
1.2	19	11.6	2.9
3.8	19	3.8	6.7
5.1	19	4.4	6.4
5.8	19	4.8	3.6
5.0	22	10.6	8.6
Average.....		8.3	8.4

TABLE IX

*Effect of  $\text{NH}_4\text{Cl}$  on the  $\text{CO}_2$  Content of Muscles Measured in Respirometers Containing Unbuffered Ringer's Solution*

Experiment No.	Time	Temperature	$\text{CO}_2$ content	
			R	N
	<i>hrs.</i>	$^{\circ}\text{C.}$	<i>cc./100 gm.</i>	<i>cc./100 gm.</i>
1	22	5	12.0	30.0
2	22	5	19.0	32.6
3	1.2	19	15.8	26.7
4	3.9	19	13.3	15.9
5	17	19	8.1	15.5
6	21	19	10.3	17.7
7	0.5	5	17.5	23.4
8	3.5	5	18.2	22.5
9	6.0	19	19.1	22.8
10	5.7	19	18.8	33.1
Average.....			15.2	24.0

The original unbuffered Ringer's solution by itself combined with no  $\text{CO}_2$ , so the increased amounts evolved must have been due to base which escaped from the muscle into the solution. The muscles previously soaked in the Ringer's solution containing  $\text{NH}_4\text{Cl}$  invariably contributed more base to the solution than the control muscle.

There is no doubt that some of the evolved  $\text{CO}_2$  in the second series was contained in the solution because we have removed the muscle before dumping the acid in some experiments and have found that the Ringer's solution remaining has acquired the ability to combine with  $\text{CO}_2$  by its contact with the muscle. In one experiment, we soaked pairs of muscles in the R and N solutions for 46 hours and then placed them in separate tubes containing unbuffered Ringer's solution through which a stream of oxygen was passing. The pH of the two solutions was measured at intervals by a glass electrode. The initial pH of both solutions was 5.2; and, 10 minutes later, it was 6.55 for the control muscles and 6.77 for the  $\text{NH}_3$ -soaked muscles. After 35 minutes, the maximum alkalinity was reached in both cases at 6.72 and 6.97 respectively. Thereafter, both solutions became progressively more acid, although the difference between them remained substantially the same.

The experiments in dry respirometers gave much more variable and less reliable results than those in which Ringer's solution was used. This was presumably due to uncertainties in the vapor pressure inside the flasks. Nevertheless, the results give no indication that the penetration of  $\text{NH}_3$  made the muscle, as a whole, any more alkaline. This is perhaps, not surprising since the pH outside was the same whether  $\text{NH}_4\text{Cl}$  was present or not.

Even in the absence of  $\text{NH}_4\text{Cl}$ , the total amount of  $\text{CO}_2$  liberated by the addition of acid is increased from 8.3 to 15.2 volumes per cent when the muscle is immersed in a solution. This is presumably due to a dilution effect. The solution, without buffers, is quite acid when it comes into equilibrium with 5 per cent  $\text{CO}_2$ , so that base tends to diffuse out of the muscle, thus equalizing the pH between the inside and the outside. In effect, the buffers of the muscle release base to form more bicarbonate to replace that lost in the solution.<sup>2</sup> This process would occur more rapidly if some of the base inside the muscle were  $\text{NH}_4$  which could diffuse more rapidly than the K. If this explanation is correct, then the experiments justify the conclusion that the pH of the muscle is not increased by penetration of  $\text{NH}_3$ , and this is consistent with the idea that the  $\text{NH}_4$  which forms inside the muscle displaces an equivalent amount of either K or Na.

Assuming a bicarbonate content of 8.4 volumes per cent after immersion

<sup>2</sup> The  $\text{H}_2\text{CO}_3$  concentration is the same in the solution as in the muscle. By adding the solution to the muscle the ratio  $\text{HCO}_3/\text{H}_2\text{CO}_3$  of the whole system is decreased. To keep the pH constant more  $\text{NaHCO}_3$  is formed from the base borrowed from proteins.

in the N solution, it is possible to estimate the pH inside the muscle. When equilibrated with 5 per cent  $\text{CO}_2$ , the solution had a pH of 6.38 and contained by calculation (at  $23^\circ\text{C}.$ ) 4.02 volumes per cent of  $\text{H}_2\text{CO}_3$ . The calculated  $\text{HCO}_3$  was then 6.53 volumes per cent which may be assumed to represent the composition of the solution in the chloride space of the muscle representing perhaps 15 per cent of the muscle. The  $\text{HCO}_3$  content of the fiber water would then be  $\frac{8.4 - (6.53 \times 0.15)}{1.00 - 0.15 - 0.20} = \frac{5.55}{0.65} = 8.5$  per cent volumes per cent, and the

pH would be  $6.17 + \log \frac{8.5}{4.02} = 6.49$ . If the pH values inside and outside

are 6.49 and 6.38 respectively, then the corresponding H ion concentrations are 3.24 and  $1.95 \times 10^{-7}$  respectively, and the  $\text{H}_i/\text{H}_o$  ratio is 1.66. If now the concentration of  $\text{NH}_3$  in the muscles after 5 hours is taken to be 22.8 m. eq. per kilo (100 per cent volume of distribution), then the amount in the extracellular space will be 3.4 m. eq., and the concentration in the fiber water will be  $(22.8 - 3.4)/(1.0 - 0.15 - 0.20)$  or 29.9 m.eq. per kilo. This is 1.31 times the concentration in the solution. Thus  $A_i/A_o = 1.31$  ( $A$  = ammonia) while  $\text{H}_i/\text{H}_o = 1.66$ . Although these calculations are based upon many doubtful assumptions (such as the correction for the extracellular space), this approximate agreement must be regarded as offering some support to the theory that the pH will determine the amount of  $\text{NH}_3$  which can accumulate inside. If this theory is correct, then the additional  $\text{NH}_3$  which accumulates in the muscles between 5 hours and 5 days' time (Fig. 2) must be due to a progressive increase in the acidity inside the muscle.

#### 10. Injection of $\text{NH}_4\text{Cl}$ into Cats

In three experiments, we have injected  $\text{NH}_4\text{Cl}$  intravenously into anesthetized cats with the idea that, under these conditions also, the  $\text{NH}_4$  would exchange with K from the muscles and cause an increase in the concentration of K in the blood plasma. At varying intervals after the injection, the plasma was sampled and analyzed for K. On account of the rapid diffusibility of  $\text{NH}_3$  it may be assumed that this disappears rapidly into the tissues in exchange for K. This K, in turn, might behave like an equivalent amount intravenously injected. As an approximation, therefore, one might calculate the volume of distribution of this hypothetically exchanged K in percentage of the body weight by the formula (mm  $\text{NH}_4\text{Cl}$  injected)  $\times 100$  divided by (K in mm per liter of plasma  $\times$  body weight in kilos).

In Table X are listed details of these three experiments with the corresponding values of the volumes of distribution calculated in this way. It will be seen that after 14 minutes the volume of distribution is 212, and this large value may be attributed to some formation of urea. According to the assumptions made, the other two values obtained after 3 and 4 minutes are not



unreasonable, since it has been shown that injected potassium is distributed in all of the body water or about 70 per cent of the body weight (Fenn, 1939, and others). In any event, this experiment shows that potassium is mobilized when  $\text{NH}_4\text{Cl}$  is injected. Subsequently, the urea content of the blood increases as Kaprowski and Uninski (1939) have shown and the excess K presumably disappears.

TABLE X

Exchange of K and  $\text{NH}_4$  in cats. The increase in the concentration of K in the blood plasma after intravenous injection of  $\text{NH}_4\text{Cl}$  under dial anesthesia.

Experiment No.	Body weight	$\text{NH}_4\text{Cl}$ injected	Time after injection	$\Delta\text{K}$ in plasma	Vol. of distribution
	kg.	mols	min.	mM per l.	per cent
1	2.9	1	3	+0.41	84
2	3.0	3	14	+0.47	212
3	3.5	2	4	+1.56	37

## DISCUSSION

Within 5 hours after immersing a frog muscle in a Ringer solution containing  $\text{NH}_4\text{Cl}$  in place of some of the  $\text{NaCl}$  the  $\text{NH}_3$  has reached a concentration inside the muscle which is equal to that in the surrounding solution. After several days, the inside concentration may be 1.5 times as great as that outside. Allowing for 20 per cent dry weight in the muscle, the concentration in the muscle water may be calculated to be respectively 1.25 to 1.9 times as concentrated as in the solution. Allowing further for an extracellular space, the concentration inside the muscle fibers themselves would be still higher. The corresponding inside to outside ratio for the potassium concentration is more like 40, depending upon the concentration of the potassium in the Ringer solution.

If the distribution of electrolytes in muscle is determined by a membrane equilibrium, as for example in the theory of Boyle and Conway (1941), then the ratios  $A_i/A_o$  (for ammonia),  $K_i/K_o$  (for potassium), and  $H_i/H_o$  (for hydrogen ions) should all be equal. Boyle and Conway have argued that the K and H ratios are indeed equal under normal conditions in muscle, but this requires that the pH inside the muscle should be 5.6. Unfortunately, the measurements of the bicarbonate content of muscle equilibrated with a known  $\text{CO}_2$  tension without previous soaking in any solution have shown that the pH is 6.8 to 6.9 (Fenn and Maurer 1935). Boyle and Conway have chosen to ignore these measurements and have selected, instead, a colorimetric determination made by Rous (1925) in muscles which might well have been injured in the process. We are unable to concede, therefore, that the inside to outside K and H ratios are equal in normal muscle.

Recently, Conway, O'Brien, and Boyle (1941) have published a brief report

of some experiments on the penetration of  $\text{NH}_3$  into muscle from very dilute solutions. Their results agree very well with our own in showing  $A_i/A_o$  ratios varying from 1.28 to 1.5. The authors reconcile this with their theory by pointing out that the potassium diffuses out until its inside-to-outside ratio is also only a little above unity. They believe, therefore, that the  $\text{NH}_3$  has changed the membrane in such a way that it requires a lower inside-to-outside cation ratio than normal. This argument, however, is rather weak when it is realized that eventually the inside-to-outside K ratio must necessarily equal 1.0 when the muscle is completely dead, so that, at some time, the  $\text{NH}_3$  and K ratios must be equal whether there is a true membrane equilibrium or not. Since, however, the K and H ratios are not equal normally, there seems no reason to suppose that the K and A ratios will be equal either. It is noteworthy, however, that the  $\text{NH}_3$  and H ratios are not far from equal in our experiments. According to the experiments of Netter (1934), the  $A_i/A_o$  ratio in perfused frog muscle is 4.5, and this is taken as a measure of the  $H_i/H_o$  ratio. Even this somewhat larger ratio is not equal to the  $K_i/K_o$  ratio.

Since the K content of muscle changes very slowly, it is possible to devise experiments which will make the  $K_i/K_o$  ratio almost anything which is required for a theory by simply changing the value of  $K_o$ . Having tried many experiments of this type, we are convinced that this ratio has no theoretical value under experimental conditions in excised muscles. It is still more difficult to make the facts fit the theory if Na is also regarded as a penetrating ion. In that case, some "pump" theory like that of Dean (1941) is required to explain the facts.

We have not been able to do a complete electrolyte balance on the same muscle, but we have shown in different muscles that both K and Na decrease in the muscle as  $\text{NH}_3$  increases, and the magnitudes are such that the combined losses of K and Na are nearly equal to the gain in  $\text{NH}_3$ . It appears likely, therefore, as a first approximation, that the  $\text{NH}_3$  enters in exchange for K and Na. It is possible, of course, that the ammonium enters as  $\text{NH}_4$  and then dissociates into  $\text{NH}_3$  ions. If this occurs, as Osterhout has pointed out, the pH should first increase as the  $\text{NH}_3$  accumulates, but it should decrease again as soon as the K and Na diffuse out. A change of this sort was actually demonstrated in *Valonia*. The evidence which we have presented for muscles indicates that any increase in alkalinity due to penetration of  $\text{NH}_3$  was minimized by an equal loss of K or Na, so that the bicarbonate content of the muscles at constant  $\text{CO}_2$  tension was not increased.

The theory of the penetration of  $\text{NH}_3$  into cells has been clearly presented by Jacobs (1927), who postulates that it is the products of hydrolysis of the salt, the  $\text{NH}_3$ , and the HA which actually penetrate. The muscle cell, according to this scheme, behaves as if only the  $\text{NH}_3$  were able to penetrate. It is reasonable to expect, therefore, that penetration of ammonia into muscle cells would be quite independent of the nature of the anion, and this seems to

be the case. The acetate was used in place of the chloride in one experiment without changing the speed of penetration. Substitution of bicarbonate buffers for phosphate buffers and equilibration with 5 per cent  $\text{CO}_2$  in place of pure oxygen did not change the result. The absence of swelling of the muscle indicates that the anion does not penetrate along with the  $\text{NH}_3$  of the  $\text{NH}_4$  ion. In this respect, the muscle behaves like the eggs of *Arbacia* (Jacobs and Stewart, 1936). In erythrocytes, which are permeable to chloride, there is a rapid swelling which may be sufficient to cause hemolysis (Jacobs, 1927). It is impossible to tell from the results whether the ammonia penetrated in ionized or non-ionized form because the end result would be the same either way. Presumably the concentration of  $\text{NH}_3$  is the same both inside and outside, in which case the total amount present (as  $\text{NH}_3$ ,  $\text{NH}_4\text{OH}$ , and  $\text{NH}_4$ ) depends upon the pH. The ability of dead muscles to concentrate  $\text{NH}_4^+$  presumably indicates that the activity coefficient is diminished inside the muscles.

#### SUMMARY

1. A study was made of the electrolyte changes which occur when frog muscles are immersed in a Ringer solution with 1/5 of the Na replaced by  $\text{NH}_4\text{Cl}$ . Analyses were made in the solution and in the muscles for K and  $\text{NH}_3$ , and the muscles were also analyzed for Cl,  $\text{HCO}_3$ , and Na. Control muscles were immersed in normal Ringer's solution and similarly analyzed.

2. The amount of ammonia taken up was about equal to the K and Na lost. There was also a small increase in chloride content. The bicarbonate content was the same in both experimental and control muscles, indicating no change in the muscle pH due to the  $\text{NH}_3$  which penetrated. An increased loss of K due to the penetration of  $\text{NH}_3$  was also demonstrated by the use of radioactive K.

3. After 5 hours, the concentration of ammonia per gram of muscle is about the same as the concentration in the solution. After 4 or 5 days, the concentration in the muscle is about 1.5 times that in the solution. The inside to outside  $\text{NH}_3$  ratio is about equal to the corresponding H ion ratio, but is much less than the K ratio.

4. The rate of penetration of the  $\text{NH}_3$  is increased by a rise of temperature, by stirring the solution, and by decrease in the concentration of Na, K, Ca, or Mg in the solution; it is decreased by increasing the size of the muscles or by killing them with chloroform or boiling.

5. Liver, smooth muscle, skin, and kidney, in a few experiments, behaved much like muscle except that there was a formation of urea in the case of liver.

6. The injection of  $\text{NH}_4\text{Cl}$  into anesthetized cats causes an increase in the level of K in the blood plasma.

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# THE RHEOLOGY OF THE BLOOD. III\*

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## *Colloidal versus True Solutions*

Rheology has not played any very important rôle as yet in either physiology or medicine. This is not because the circulation is unimportant or thoroughly understood. It is rather because of the failure of the simple law of Poiseuille to represent the complex conditions of flow in the blood and other colloidal solutions (1). The invention of the terms "structure viscosity" (2) and "deformation elasticity" (3) seemed to attribute the difficulty to the colloidal character of the solutions requiring a knowledge of the laws of flow of soft solids or plastic bodies. But even a more fundamental mistake has been made in assuming that viscosities are additive in mixtures. In a mixture of simple liquids, the fluidity may not be that given by the additive law in any given case because there are several factors, such as solvation or dissociation which can interfere, but it still remains valid (4) that the fluidities are additive in homogeneous liquid mixtures. In this and succeeding papers we shall, by the use of the literature and our own experiments, attempt to show in a manner not attempted heretofore how the rheological properties of the blood can be usefully correlated. We shall at first regard the blood as a viscous liquid because that is simpler, it is not grossly incorrect, and it enables us to make the maximum use of the earlier work.

In homogeneous mixtures of inert liquids, we assume the fluidities to be additive, and not viscosities, thus

$$\Phi = a\Phi_1 + b\Phi_2$$

or

$$= \Phi_1 + b(\Phi_2 - \Phi_1) \quad (1)$$

where  $a$  and  $b$  are the volume fractions of the components  $A$  and  $B$ , whose respective fluidities are  $\Phi_1$  and  $\Phi_2$ . For colloidal solutions, it was found first for suspensions (5) and then for sols that the fluidity (6) is linear still but approaches a value of  $b$  which is no longer unity, but  $b'$ , so that

$$\Phi = (1 - b/b')\Phi_1 \quad (2)$$

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\* Bingham, E. C., and Roepke, R. R., The rheology of the blood, unpublished paper presented before the Society of Rheology. The effect of fibrinogen on the fluidity of blood plasma, *J. Am. Chem. Soc.*, 1943, **64**, 1204.

This equation was first applied to clay, graphite, sulfur sol, silver sol, gelatin, and sodium palmitate, etc. Treffers (7) has extended the principle of additive fluidities to various protein solutions. He concluded that "in addition to its advantages of simplicity and ease of application, it fits available data quite well . . . . The fluidity is linear with the protein concentrations over a wide range of proteins and concentrations."

#### *Absolute versus Relative Fluidities*

In giving data as absolute fluidities in rhes, an explanation is required for the departure from the usual custom of using *relative* values only. (1) The use of relative fluidity, defined as the ratio of the fluidity of blood to the fluidity of water at 20°, would not give a quantity which can be operated upon mathematically. (2) W. R. Hess (8) who was largely responsible for the adoption of the use of "relative viscosity" did not use water at a fixed temperature as standard, but rather at *the temperature of measurement*. This adds the disadvantage that the standard is not a fixed standard and to a degree the standard is uncertain. The example of Hess has been generally followed. (3) Hess (8) claimed that the relative fluidity as defined by him is independent of the temperature. The result has been that the word "relative" is often omitted from the data and the temperature of measurement is not mentioned. The claim of Hess may be true, or nearly true, but the use of relative fluidities would tend to conceal its significance, for two different liquids cannot be assumed *a priori* to have the same temperature coefficient of fluidity. Thus for example, water and mercury have the same fluidity of 60 rhes at 2.1° C. but at 20°C. the fluidity of mercury is 64.3 while the fluidity of water is 99.5 rhes, so that the relative fluidity at 20° is no longer unity but 0.646. The answer to the question as to why the Hess assumption could be made for blood has never been given, but of that later (p. 84). (4) Actually Hess recommended the measurement of the viscosity of the blood at room temperature as a matter of convenience, but the physiologist is primarily interested in the blood at its "working temperature." For example, Burton-Opitz (9) recognized that the temperature of the living blood is the temperature at which to compare different animals and then absolute fluidities become significant, the frog with the low working temperature having approximately the same fluidity of blood as the mammal at 37°. (5) But this virtual disregard of the temperature in recording only relative fluidities would virtually assume as a fact that different samples of blood are alike, so that cooling one blood down to room temperature might actually have an effect somewhat different from that on the ordinary or normal blood. It is not necessary to be specific as to whether the result is a coagulation, rouleaux formation, or crystallization. It seems an unnecessary complication to be avoided if possible. (6) Finally, in tables

of constants, it is confusing to have data based on several standards, particularly since we do not have even one standard of unquestioned accuracy, water at 20°C.  $\Phi = 99.5$  rhes.

Nevertheless, many data in the literature have been converted to fluidities and compared. We will study first the data of Nägeli (10) on the viscosity of human blood serum. The data as taken from Bircher (11a) are given in Table I, but the obviously incorrect value of 1.57 has been changed to 1.51 as a probable typographical error. Since the measurements were made at about 20°, the fluidity has been calculated for that temperature (column 3); but we have also calculated the fluidity of the blood serum at 37° (column 4),

TABLE I  
*The Relation of the Fluidity of Human Blood Serum to the Protein Content*  
After Nägeli (10).  $\Phi_{20^\circ} = 89.8 - 3.99 b$ ;  $\Phi_{37^\circ} = 129.6 - 5.72 b$

Protein	Relative viscosity	Fluidity at 20°	Fluidity at 37°	Fluidity at 20° equation (2)	Fluidity at 37° equation (2)
<i>per cent</i>		<i>rhes</i>	<i>rhes</i>		
5.0	1.43	69.6	100.7	69.8	101.0
5.5	1.46	68.2	98.6	67.8	98.2
6.0	1.51	65.9	95.4	65.8	95.3
6.5	1.56	63.8	92.3	63.8	92.4
7.0	1.61	61.8	89.4	61.9	89.6
7.5	1.67	59.6	86.2	59.9	86.7
8.0	1.72	57.8	83.7	57.9	83.8
8.5	1.78	55.9	80.9	55.9	81.0
9.0	1.84	54.1	78.3	53.9	78.1
9.5	1.90	52.4	75.8	51.9	75.3

on the assumption of the relative viscosity (or fluidity) being independent of the temperature. Applying equation (2) to these data, the constants  $b'$  and  $\Phi_1$  have been obtained and the fluidities given in the last two columns (5 and 6) of the table computed. They agree well with the "observed" values of columns 3 and 4 respectively the average deviation being nearly 0.3 per cent for each case. The fluidity curves are therefore linear. At 20° the value of  $b$  where  $\Phi = 0$  is 0.225 and at 37° it is 0.226, which may be considered identity, but the fluidity of the medium at zero concentration of protein turns out to be  $\Phi_1 = 89.8$  rhes at 20° and 129.6 rhes at 37°C. The non-protein medium has a fluidity considerably lower than water, 99.5 at 20° and 144 at 37° the difference amounting to nearly 10 per cent.

For a second study, it is interesting to use data measured by Hess (13) in 1906 obtained by measuring the fluidities of mixtures of human blood serum with different amounts of physiological salt solution. He obtained a strongly hyperbolic curve which was confirmed with the serum of other men and ani-



mals. He was apparently puzzled, because others working under his direction reverted to the same problem (Blunschy (14) and Bircher (11*b*)) and they discussed the fact that the curve is non-linear. It did not occur to them to use fluidities which are linear, as shown in Table II. The experiments of Bircher (11) with sheep blood serum are more precise and numerous, hence

TABLE II  
*The Fluidity of Human Blood-Serum and Physiological Salt Solution*  
After Hess (13).  $\Phi_{37^\circ} = 115.8 - 0.936 b$

Volume	Relative viscosity	Fluidity at 37°	Fluidity at 37°, equation (2)
<i>per cent</i>		<i>rhes</i>	
20	1.4	102.8	97.1
40	1.8	80.0	78.4
60	2.75	52.4	59.7
80	3.8	37.9	41.0
100	5.7	25.3	22.2

TABLE III  
*The Relation of Fluidity to the Protein Content of Sheep Blood Serum*  
After Bircher (11)

Volume of serum	Protein	Bircher relative viscosity corrected to 15°	Fluidity at 20° observed	Fluidity at 20° calculated
	<i>per cent</i>		<i>rhes</i>	<i>rhes</i>
10	0.7	1.05	95.2	94.3
20	1.4	1.10	90.1	90.4
30	2.1	1.16	86.2	86.5
40	2.8	1.22	82.6	82.5
50	3.5	1.28	78.4	78.6
55	2.85	1.32	76.3	76.7
60	4.2	1.35	74.1	74.7
65	4.55	1.405	72.5	72.8
70	4.9	1.435	70.9	70.8
80	5.6	1.53	66.7	66.9
90	6.3	1.63	63.3	63.0
100	7.0	1.74	59.5	59.0

they have been employed in Table III. Bircher used the serum of several different animals which he mixed with 0.95 per cent saline solution. The fluidities have been computed from his data for 20° and the formula fitted to the data is  $\Phi = 98.2 - 5.60 b$ , where  $b$  is the fraction of protein present. With  $\Phi_1 = 98.2$ , which is the value expected for physiological salt solution, the concentration required for zero fluidity; *i.e.*,  $b' = 0.176$ . The percentage deviation given by the formula is 0.4 per cent.

TABLE IV

*The Fluidity of Mixtures of Human Blood Plasma with Sediments in Plasma Containing 6,616,000 Erythrocytes per mm<sup>3</sup>.*

After Blunschy (14).  $\Phi = 63 - 52.3 b$

Volume	Relative viscosity 19°	Fluidity at 37° observed	Fluidity at 37° calculated
<i>per cent</i>		<i>rhes</i>	<i>rhes</i>
0	2.27	63.5	63.0
25	2.87	50.1	50.2
50	3.92	36.7	37.2
75	6.25	23.0	24.3
100	11.4	12.6	11.4

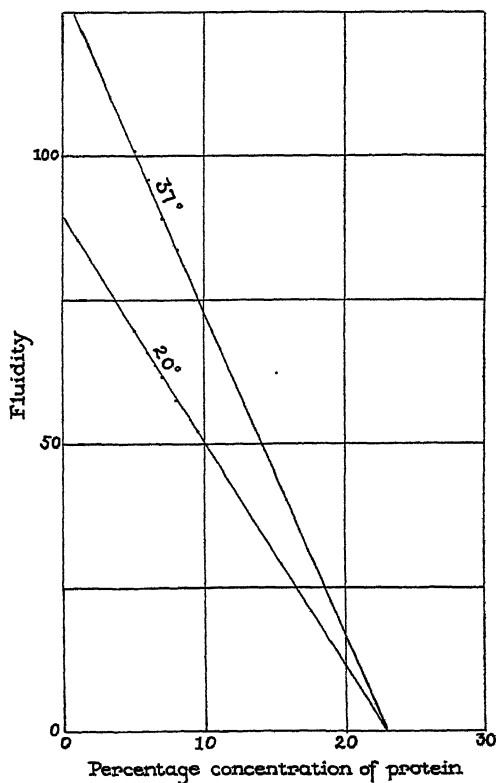


FIG. 1

Having shown that equation (2) applies to many types of colloidal solutions including proteins and even blood serum, it is desirable to include data (Table IV) from Blunschy, page 10, on suspensions of human red blood corpuscles in plasma. The plasma was obtained by centrifuging and the mixtures made

by the addition of the plasma to a sediment containing 6,616,000 corpuscles per mm.<sup>3</sup> The measurement was made at 19° and the blood was venous. The fluidity of the plasma at 37° is  $\Phi_1 = 63.0$  and the concentration of zero fluidity is  $b' = 1.02$ , which signifies that the sediment contained enough plasma so that it behaved as a viscous liquid; nevertheless the sediment gives a fluidity which is 12.6, which is much too far away from the calculated value of 11.4 rhes. The average percentage deviation is only 0.7. In passing it is worth noting that this blood was taken from a patient suffering from croupous pneumonia and may therefore have had a considerable tendency to coagulate, with a high sedimentation rate. These results which seemed inexplicable

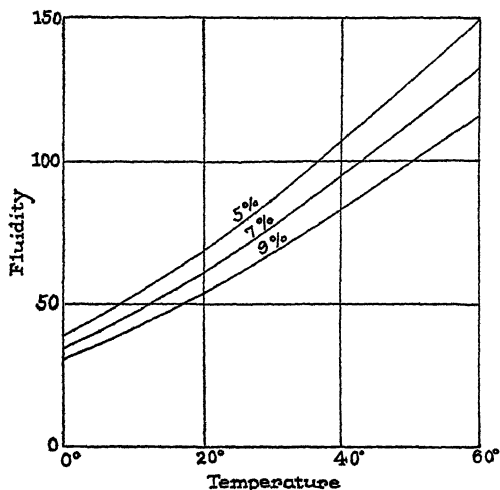


FIG. 2

to Blunschy, seem normal enough now. But if a sediment with much less plasma were used, we may be sure that problems of plastic flow would enter.

#### *The Effect of Temperature on the Fluidity of Protein Solutions*

We now revert to the consideration of the rule of Hess that the relative fluidity (or viscosity) of blood is independent of the temperature, at least within certain specified limits but they have often been disregarded. The fluidity of human blood serum as given by Nägeli for various concentrations of protein in Table I has been plotted in Figs. 1 and 2, the fluidity *versus* concentration and temperature respectively. The linear character of the fluidity concentration curve is a demonstration of the applicability of the rule of Hess to human blood serum: it is also in accordance with the law of fluidity of colloidal suspensoids, equation (2), and we get

$$\frac{\Phi_{i1} c_0}{\Phi_{i2} c_0} = \frac{\Phi_{i1} c_1}{\Phi_{i2} c_1} \quad (3)$$

where  $\Phi_{t_1 c_0}$  represents the fluidity at temperature  $t_1$  at zero concentration, etc., and according to the rule of Hess

$$\frac{d\Phi}{db} = -[K]_t = \text{concentration coefficient of fluidity,}$$

hence on integration,

$$\Phi = C - [K]_t b \quad (2a)$$

where  $C = [\Phi]_{c=0}$ , *i.e.*, the fluidity of the medium when the concentration is zero. Also  $[K]_t = \Phi_1/b'$ .

From equation (3) it follows that

$$\frac{\Phi_{t_1} c_1}{\Phi_{t_1} c_0} = \frac{\Phi_{t_2} c_1}{\Phi_{t_2} c_0} = [K]_b$$

then

$$\frac{d\Phi}{dt} = [K]_b = \text{temperature coefficient of fluidity}$$

and

$$\Phi = [K]_b t' + D \quad (4)$$

where  $D$  is the fluidity at temperature zero,  $[\Phi]_{t=0}$  and  $[K]_b = -[\Phi]_{t=0}/t'$ . As the temperature is lowered,  $t'$  is the temperature at which the fluidity becomes zero.

One cannot assume that the above relation will apply exactly in a given case. The law is valid for the ideal case, and therefore it is the deviations from the law that engage the greatest interest. The law itself merely states that the fluidity of a suspension depends solely upon the fluidity of the volume of medium which is present. This suggests at once that if some of the medium is immobilized by being absorbed into porous particles or if the particles are partially dissolved in the medium or if aggregates are broken down on shearing, the application of the law will show deviations. This matter is of such importance that a case in point will now be considered, using the abundant data of Jacques Loeb (15) on isoelectric gelatin up to 4 per cent gelatin<sup>1</sup> at temperatures from 25 to 60°.

We have converted the relative viscosities to rhes given in Table V and we have plotted the data in Fig. 3. Loeb noted that "at 25° the agreement is satisfactory only at the lowest concentrations. . . . The gelatin solidifies so rapidly that viscosity measurements were no longer possible for a concentration of 3.5 per cent." The curves are all linear and all except the data for 25° converge at  $-74$  rhes, and in view of Loeb's remarks we reserve any

<sup>1</sup> Loeb, page 204, gives the data under the wrong heading " $\log \eta/\eta_0$ " instead of " $\eta/\eta_0$ " in the original paper, *J. Gen. Physiol.*, 1919, **1**, 483.

TABLE V

*The Fluidities of Solutions of Isoelectric Gelatin in Water at Various Concentrations and Temperatures*

After J. Loeb and calculated by the formula:  $\Phi = (1 - 0.0659 b) (\Phi_1 + 73.9) - 73.9$

Concentration	$\Phi_{25}^{\circ}$ Observed	$\Phi_{25}^{\circ}$ Calculated	$\Phi_{35}^{\circ}$ Observed	$\Phi_{35}^{\circ}$ Calculated	$\Phi_{45}^{\circ}$ Observed	$\Phi_{45}^{\circ}$ Calculated	$\Phi_{60}^{\circ}$ Observed	$\Phi_{60}^{\circ}$ Calculated
<i>vol. per cent</i>								
0	(111.9)	110.3	(138.4)	135.1	(167.0)	163.1	(213.3)	212.1
0.25	107.9	107.3	134.8	131.8	162.0	159.3	208.4	207.6
0.50	103.7	104.4	129.6	128.5	156.3	155.6	203.1	203.0
1.0	95.8	98.1	120.6	121.3	147.1	147.5	195.2	193.3
1.5	87.7	92.7	111.9	115.1	137.6	140.4	183.0	184.8
2.0	81.7	86.8	106.0	108.5	130.5	132.9	172.7	175.7
2.5	76.2	81.0	100.2	101.8	123.6	125.4	164.7	166.6
3.0	66.1	75.1	93.3	95.2	115.9	117.8	162.9	157.4
3.5	—	—	89.2	88.5	111.0	110.3	149.0	148.4
4.0	—	—	86.3	81.8	106.6	102.7	140.2	139.2
Average deviation, per cent. ....				2.2		1.5		0.9

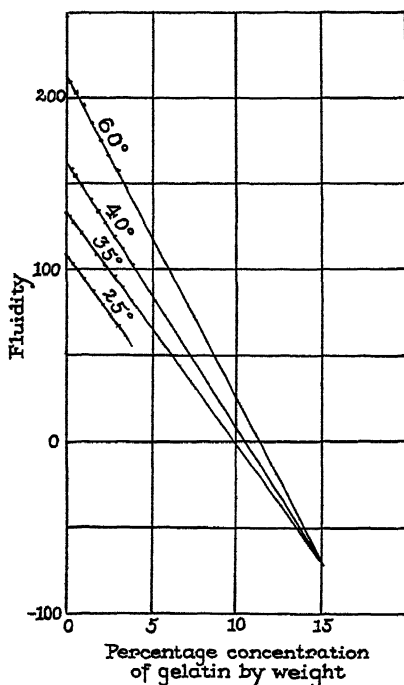


FIG. 3

judgment in regard to that exception. The fact that the curves differ from all suspensions heretofore studied in not converging at zero fluidity signifies that the temperature of zero fluidity falls as the temperature of the gelatin solutions studied is lowered, and it can be explained, since gelatin is a lyophilic colloid and known to form hydrates, by the gelatin absorbing an increasing amount of water as the temperature is lowered. This necessitates adding a parameter to equation (2), which becomes

$$\frac{\Phi - c}{\Phi_1 - c} = 1 - 0.0659b \quad (2b)$$

where  $c = -73.9$ . The fluidities calculated by this for the various temperatures and concentrations are all given in the table. The average deviation (1.5 per cent) between the observed and calculated fluidities increases some-

TABLE VI  
*Constants for 35°, 45°, and 60°, in Equation (2c) for Isoelectric Gelatin*

Temperature	Constants			
	$\alpha$ observed	$\alpha$ calculated	$\beta$ observed	$\beta$ calculated
°C.				
35	135.1	138.4	13.35	10.12
45	163.1	167.0	15.21	10.73
60	212.1	213.3	18.06	11.74

what as the temperature is lowered. In obtaining the equation (2b) we first obtained the best linear equation of the form of equation (2)

$$\Phi = \alpha - \beta b \quad (2c)$$

These constants are given in Table VI together with the fluidity of water at the same temperatures. The lowest volume percentage of gelatin is 0.25 and it gives no indication of lack of linearity, yet this may be best explained by a portion of the gelatin dissolving in the water to form a true solution, thereby lowering the fluidity.

As the value of  $\beta$  in equation (2c) or  $\Phi_1/b'$  in equation (2) is a function of the temperature, it seems highly desirable for our further study in human physiology to have a standard temperature for the comparison of all proteins, 37° being very convenient as well as logical. In the case of gelatin  $\Phi - c$  is used in place of  $\Phi$  in equation (2b) and thus a value for  $\beta$  agreeing closely for the three temperatures of 35°, 45°, and 60° is obtained of 13.72. This is then a fairly definite and reproducible rheological constant for proteins and perhaps other types of colloids. Since the rheological properties of colloids vary

within wide limits and are very important as well as characteristic, such a constant may be of value.

Before leaving the consideration of gelatin, it is noted that the concentration of zero fluidity rises with the temperature and therefore at some concentration may reach unity; equation (2) then becomes

$$\Phi = (1 - b)\Phi_1$$

and since  $1 - b = a$   
therefore

$$\Phi = a\Phi_1 \quad (2d)$$

This critical equation is so called because it is the transition between true and colloidal solutions, between equations (1) and (2). It is the analog of the law

TABLE VII  
*The Fluidity-Temperature Coefficient of Whole Blood*  
After Kagan (12)

$\eta$ relative 37° observed	$\eta$ relative 17° observed	$\Phi$ 37° observed	$\Phi$ 17° observed	$\Phi$ 17° calculated
3.8	4.6	37.9	31.3	31.4
4.0	4.85	36.0	29.7	29.8
3.08	3.7	46.8	38.9	38.8
4.25	5.1	33.9	28.2	28.1

of Raoult. True solutions are viscous whereas at shearing stresses below the yield stress, colloidal solutions are plastic.

#### *The Fluidity-Temperature Coefficient*

Kagan (12) made measurements of the relative viscosity of whole blood at both 17° and 37° from four individuals with the special purpose of checking the statement of Hess that this lowering of the temperature of the blood caused a 16 per cent increase in the relative viscosity or 0.8 per cent per degree. We give the scanty data in Table VII for what they are worth realizing that measurements at three or more temperatures are necessary if we are to learn how the fluidity varies with the temperature. The observed relative viscosities are given in the first two columns and the computed fluidities in rhes in the third and fourth columns, and the calculated fluidities at 17° in the fifth column, using the following formula:

$$\Phi = (1 - \gamma\Delta t)\Phi_{17} \quad (2e)$$

which is a form of equation (2), where  $\gamma$  is the fluidity-temperature coefficient per degree from 37°, the temperature difference being  $\Delta t = 20^\circ$ . From the

observed fluidities, the average value of  $\gamma$  is found to be 0.86 instead of simply 0.8 and the agreement between the observed values and those calculated by the formula are all that can be expected from the data.

In an ideal suspensoid in water, the fluidity-temperature coefficient should be a fraction of that of the medium itself, which can be best obtained by differentiation of the fluidity-temperature equation. But we may say that calculated in the same way over the same temperature interval, water gives a value for the fluidity-temperature coefficient which is 1.79 which is double the value accepted for blood. The obvious explanation, that the medium in the case of blood is not pure water, is hardly satisfying. The question needs looking into. Fig. 2 proves that the fluidity-temperature curves are not linear.

### *The Fluidity of a Mixture of Proteins*

If the fluidity of a mixture of protein solutions is to be calculated from the concentration coefficients of the individual proteins, more information is required than may be available at present. Many of the data now available are for one temperature only, so that the estimation of the hydration is not practicable. Fortunately, the blood proteins are apparently but little hydrated. For example, Kunitz, Anson, and Northrop (16) report that the hydration of gelatin is forty-five times greater than that of hemoglobin. We can, however, calculate the concentration coefficient for the temperature of the blood  $\beta_{37^\circ}$  on the assumption that equation (2) applies, which has been done for over twenty proteins (Table VIII). There are two difficulties further, the one that concentrations are often based on weight instead of volume; another embarrassment is due to the possibility that the solvent medium is not pure water. This becomes a certainty in the cases of globulin and fibrinogen, since they dissolve in salt solutions only, but a physiological salt solution has a fluidity of only about 1 rhe below that of water. And it goes without saying that the pH of the medium needs careful consideration. Table VI shows that the average deviation between the values of  $\alpha$  and  $\Phi_1$  is only 1 per cent. Dissolved substances would nearly all decrease the fluidity but the lowering exceeds the raising of the fluidity by only 0.1 per cent which is indeed negligible. The values of  $\beta_{37^\circ}$  are arranged in decreasing order.

If in the blood the various salts and proteins are present in concentrations,  $b_1$ ,  $b_2$ ,  $b_3$ , etc. with values of the concentration-coefficients of  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ , etc., then on the hypothesis that fluidities are additive

$$\Phi = \Phi_1 - \beta_1 b_1 - \beta_2 b_2 - \beta_3 b_3 - \dots \quad (5)$$

or the lowering of the fluidity of the blood at  $37^\circ$   $\Phi - \Phi_1 = \Delta\Phi$   
and

$$\Delta\Phi = \beta_1 b_1 + \beta_2 b_2 + \beta_3 b_3 + \dots \quad (6)$$



where the first term on the right side represents the total effect of the salts 1.3 rhes, the second term that of the albumin of perhaps 24.4 rhes, the third that of globulin, the two together amounting to perhaps 56.2 rhes and the fourth that of fibrinogen, 16.2 rhes. The average values for the proteins in human blood given by Lewinski (17) are 4.01 serum albumin, 2.83 serum globulin,

TABLE VIII  
*The Fluidity-Concentration Coefficients ( $\beta_{37}^{\circ}$ ) for Various Protein Solutions*

Protein	$\alpha$	$\Phi$	$\beta \times 100$	$\beta_{37}^{\circ} \times 100$
Globulin (20).....	115	114	2237	2801
Globulin (beef) (20).....	117	114	785	965
Fibrinogen (dog)*.....	112	112	26.4	33.9
Globulin (horse) (21) III.....	114	112	23.8	29.8
Globulin (horse) (21) II.....	115	112	16.6	20.8
Globulin (horse) (21) I.....	113	112	11.6	10.43
Globulin (horse) (21) sample 2.....	109	112	10.4	13.77
Gelatin, isoelectric (14).....	135	138	13.4	13.72
Gliadin (22).....	99	99.5	9.93	13.02
Pseudoglobulin (21).....	112	112	8.99	11.60
Octopus hemocyanin (22).....	97.6	99.5	5.39	7.95
Trypsin (16).....	65.7	65.8	3.30	7.23
Albumin (horse) (21).....	111.7	111.9	5.13	6.61
Albumin, sample 2.....	110.4	111.9	4.80	6.26
Amandin (22).....	98.5	99.5	4.38	6.40
Serum albumin (22).....	98.7	99.5	3.94	5.76
Serum albumin (10).....	130	144	5.72	5.72
Lactoglobulin (22).....	98.5	99.5	3.76	5.50
Ovalbumin (22).....	98.9	99.5	3.76	5.49
CO-Albumin (22).....	99.3	99.5	3.75	5.43
CO-Albumin (16).....	66.1	65.8	2.40	5.24
Egg albumin (14).....	87.8	87.6	1.41	2.32
Oxyhemoglobin (24).....	112.4	111.9	1.80	2.28

\* Bingham, E. C., and Roepke, R. R., The rheology of the blood, unpublished paper presented before the Society of Rheology. The effect of fibrinogen on the fluidity of blood plasma, *J. Am. Chem. Soc.*, 1943, **65**, 1204.

and 0.42 fibrinogen, reckoned as grams per 100 cc. of plasma. From the above  $\Delta\Phi$  turns out to be 73.7 rhes and therefore the fluidity of blood plasma would be  $144.0 - 73.7 = 70.3$  rhes. This accords well with the average value.

We have not yet considered the effect of erythrocytes, leukocytes, and platelets on the fluidity of the blood. That subject will be considered in the next paper. The plasma and serum follow the law of Poiseuille without question so exact constants can be obtained, at least in theory. But with albumin, globulin, and fibrinogen qualified by the source and perhaps other conditions,

the identity of the substances studied here appears not well defined, hence much work is needed in confirming the earlier measurements. To give added point, it is noted that the data on protein solutions of a few observers deviate widely from the linear curve. We have not been able to fit a linear curve to the data by Chick (18) for pseudoglobulin or for egg albumin by Chick and Lubrzenska (19). This seems less important since other data on these same substances by different observers do yield linear fluidity-concentration curves. It is hoped that these irregularities may be explained.

#### CONCLUSIONS

1. The fluidity-concentration equation (1) for true solutions and the corresponding equation (2) for suspensoids and suspensions merge into each other in equation (2*d*),  $\Phi = \alpha\Phi_1$ , where  $\Phi_1$  is the fluidity of the medium *A*, of which  $\alpha$  is the volume fraction present. Equation (2*d*) defines the critical relation between colloidal and true solutions and is the analog of Raoult's law.

2. The importance of not only using fluidities but also of using absolute fluidities (rhes) and not relative fluidities is emphasized.

3. The rule of Hess, that the fluidity (or viscosity) of blood, serum, or plasma is independent of the temperature was based on observation but without theoretical explanation. The above law of fluidity of suspensoids that the fluidity-concentration curves are linear, coupled with the observation that the concentration at which the fluidity reaches zero is independent of the temperature, makes the rule of Hess follow as a necessity. The rule is true because the fluidity of a suspensoid is due solely to the volume of the medium present and its coefficient of fluidity. Therefore it is possible to predict the fluidity of such a solution at any concentration or temperature, from a knowledge of  $b'$  and that of the fluidity of the medium at the desired temperature. These relations follow as a result of the fact that the fluidity-temperature curve of water is *nearly* linear. The rule of Hess is known to be only approximately true and the same may be true of equation (2).

4. If the value of  $b'$  is a function of the shearing stress, it is probably because the material is plastic above a certain concentration and below a certain shearing stress—the yield stress. No plasticity has been detected by us in the blood plasma at the temperature and concentration of proteins found in the body. This however does not apply to whole blood.

5. With lyophilic colloids, it is found that the value of  $b'$  changes with the temperature of the solution, which is attributed to changes in solvation with the temperature. By the use of one additional parameter, it is nevertheless possible to still predict the fluidity of solutions of isoelectric gelatin from 35 to 60° with an average deviation of less than 2 per cent.

6. The fluidity of the various proteins in the plasma can be calculated from the fluidity-concentration coefficients at 37° which is a convenient standard

temperature. These coefficients can be calculated from existing data provisionally on the reasonable assumption that most of the proteins in blood, albumin, globulins, and fibrinogen are not greatly hydrated. If the fluidities of mixtures of these proteins are additive, *i.e.*, compatible, it becomes feasible to calculate the fluidity of the plasma from the fluidity-concentration coefficients and the fluidity of water, 144.0 at 37°. Whereas the result is reasonable, the constants are only tentative and they are to be used with caution.

#### SUMMARY

The authors have confirmed the fact that blood serum and plasma behave rheologically like a true viscous liquid. It is true for whole blood only to a first approximation, but with this reservation they have studied the available data and extended the equation of Bingham and Durham to cover protein solutions of various concentrations and at various temperatures as well as mixtures of proteins and corpuscles present in whole blood. If  $\Phi$  is the fluidity of whole blood,  $\Phi_1$  is the fluidity of water and  $\Delta\Phi = \Phi - \Phi_1$ , then

$$\Delta\Phi = \beta_1 b_1 + \beta_2 b_2 + \beta_3 b_3 + \dots$$

where  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ , etc., are constants for the fluidity lowering of the salts, albumin, globulin, fibrinogen, and the corpuscles, etc., present in the whole blood.

The conclusions from the data referred to are intended to buttress this simple equation (6).

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of Table I. The data represent only the *apparent* rates of photosynthesis uncorrected for respiration. Respiratory rates were not measured.

Values of the apparent rate of photosynthesis per cubic millimeter of cells (fifth and sixth columns) show, for each carbon dioxide concentration, a maximum variation of 6 to 7 per cent and a standard deviation of about 2 per cent. The experimental errors in these values are contributed both by the errors of the manometric measurement and the error in determination of cell volume.

Additional data (not shown in the table) were obtained by occasional measurement of the pH of the suspension immediately after harvesting. Measurements with a Coleman glass electrode yielded pH values lying between 6.05 and 6.10. The fresh Knop's solution used had a pH of 5.0.

#### DISCUSSION

The data of Table I on maximum apparent rate of photosynthesis describe one arbitrarily chosen physiological characteristic of the cells. It is our experience that for a culture allowed to mature along the growth curve, the maximum rate of photosynthesis may decrease to a value as low as 0.05 c.mm. O<sub>2</sub> per min. per c.mm. of cells. Similar results were obtained by Sargent (1940, Table IV). Sargent's data (Table III) also illustrate the considerable variation in maximum rate of photosynthesis to be expected for cells of different cultures harvested at approximately the same age. It is evident that the maximum rate of photosynthesis is a characteristic which may vary widely between different batches of algal cells cultured by the usual procedures. The data (columns 5 and 6) of Table I illustrate the uniformity in maximum rate of photosynthesis to be expected of cells obtained from the continuous culture apparatus. Rate of growth also shows fair uniformity. Other physiological characteristics have not been examined. However, it is reasonable to expect that cells will be equally uniform in other characteristics since they are grown under highly uniform conditions.

In only one respect has the apparatus fallen short of theoretical expectations. A basic assumption is that the sample removed and the inoculum left in the chamber are identical as to concentrations of all components (*i.e.*, cells, inorganic ions, metabolites). This condition is not entirely attained. Because of the bubbling action, surface-active materials tend to accumulate at the upper liquid-gas interface and therefore do not distribute equally between the sample and inoculum. Foaming occurs, though a permanent foam is not formed until after several weeks of operation. However, no detrimental effects of the surface-active materials have yet been observed.

The data of Table I describe the operation of the apparatus as applied to one of the two purposes for which it was designed; *i.e.*, the production of uniform experimental material day after day. Application of the apparatus in studying

the relation of culture conditions to photosynthetic behavior will be considered in later papers of this series.

#### SUMMARY

1. An apparatus has been developed which maintains a constant density of population of *Chlorella* by automatic dilution of the growing culture with fresh medium.

2. Cells harvested from the apparatus in daily samples are highly uniform in rate of growth and rate of photosynthesis measured under arbitrarily chosen conditions.

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# A SELECTIVE LETHAL EFFECT OF PENICILLIN ON SARCOMA CELLS GROWING WITH NORMAL TISSUE IN ROLLER TUBE CULTURES\*

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PLATES 1 AND 2

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The clinical use of penicillin at Walter Reed General Hospital during 1942-43 made available small amounts of this substance for experimental study. Accordingly, at the suggestion of Lieutenant Colonel Carl J. Lind and Captain Jack M. Evans, some of the drug was taken to The Wistar Institute of Anatomy and Biology in Philadelphia, for use in tissue culture experiments. The early observation of what appeared to be a specific effect on sarcoma cells (2) prompted the following study. We are indebted to Dr. Margaret Reed Lewis and Dr. Warren H. Lewis for the use of their laboratory facilities and for their help throughout these studies.

## *Material and Method*

Rats of the King A<sup>1</sup> and of the Wistar albino strains, and mice of the black (C<sub>57</sub>) and the Bagg Albino (B.A.) strains were used. Each of these inbred strains had proved to be 100 per cent susceptible to the grafts of sarcomata that had been induced in the strain (3). Six rat sarcomata (King A No. 11, No. 89, No. 104, No. 120, and No. 132 and Wistar No. 304) and two mouse sarcomata (C<sub>57</sub> No. 350 and B.A., No. 37) were used in the cultures. These spindle cell sarcomata had been induced by subdermal injection of dibenzanthracene or benzpyrene (5). The normal fibroblasts were derived from fragments of muscle from rats or mice, 1 to 2 days old, of the tumor host strain.

Roller tube cultures (4) with usually eight to ten fragments of a tumor and an equal number of muscle fragments 1 to 2 mm. in diameter were grown in a medium composed of 2 drops of chicken plasma, 2 drops of chick embryo extract, 5 drops of human placental serum, and 7 drops of Locke's saline solution. The pipettes used measured 18 to 20 drops to the cubic centimeter. Extensive outgrowth was obtained in 24 to 72 hours, and at this time a record was made of the extent of growth. The initial medium was then replaced with a medium of 7 drops of Locke's solution, 5 of serum, and 2 of plasma, and in the experimental tubes, 1 to 3 drops of penicillin solution were substituted for an equal quantity of Locke's solution. This penicillin

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\* Aided by a grant made to Dr. Warren H. Lewis from the International Cancer Research Fund. We are also indebted to The Wistar Institute of Anatomy and Biology and to the Carnegie Institution of Washington.

<sup>1</sup> Kindly supplied by Dr. Helen Dean King.



solution was prepared from Squibb or Reichel sodium salt of penicillin dissolved in 0.85 per cent sodium chloride, and filtered through a sterile Seitz filter. Such pharmaceutical penicillin preparations contain substances in addition to the penicillin.

The duration of exposure was varied, dosage usually being continued until a definite selective effect was observed. After the effect of the drug had been studied, the penicillin medium was replaced with a Locke's solution + serum + plasma medium, and the recovery processes were studied. In those instances where the injured tumor explants showed renewed growth, half the explants were implanted into an animal of the strain native to the sarcoma to test whether the viable cells were malignant or stromal cells. In all, 38 tubes, treated and untreated, were studied.

### RESULTS

The malignant cells were consistently more injured than the normal ones (Figs. 1 and 2, 4 and 5). With adequate dosage it was found possible to damage and kill the outgrowing cells of all six rat tumors and one of the mouse tumors without killing the cells which grew from the fragments of normal muscle. These malignant cells first reacted to the penicillin by assuming a granular, opaque appearance, with or without vacuoles. This was the initial response to a heavy dose of the drug or the full extent of response to a threshold dose. If the penicillin was removed at this point, all cells recovered. In the higher concentration, along with increasing granularity and darkening of the cytoplasm, there was a retraction of the elongate processes, producing cells irregularly rounded. Upon prolonged exposure the cells disintegrated. Even if penicillin was removed after the cells had rounded up, some of the cells never recovered. This sequence of changes is not peculiar to penicillin, but is the usual response to many cytotoxins and moderately toxic compounds. In the higher concentrations of penicillin, the normal fibroblasts followed the same sequence of changes. However, there was the very important difference that a concentration of penicillin sufficient to cause the rounding up of some of the fibroblasts, in most instances caused the death of all of the malignant cells. A dose too weak to produce any visible cytological changes was nevertheless selective in that it inhibited growth from the malignant explants, while growth of the normal explants was unaffected. In the untreated control tubes the outgrowth of the sarcoma equalled and usually exceeded the outgrowth of the normal cells.

To obtain a quantitative statement of the results, damage to the cells can be classified as incipient (granularity of 50 per cent or more of the cells, and increased irregularity and refractility of the cell boundary), marked damage (rounding, coagulation, or disintegration of the cells, short of 100 per cent), or lethal (no living cells visible). Table I shows the totals of explants classified according to their damage. A further subdivision of the comparisons better reveals the extent of the selective effect. In those tubes in which the 112 normal colonies were not at all affected, there were 29 of sarcoma which

showed incipient effect, 114 which showed marked damage (Figs. 2 and 5), and 23 which were dead. In tubes showing incipient effects upon 92 normal colonies, 70 tumor growths showed marked damage and 46 were completely killed. In tubes in which the 57 normal colonies showed marked damage, 24 colonies of tumor were markedly damaged, and 35 were killed. The overall effect was clear cut. Not only was the malignant tissue damaged more than the normal throughout the series, but in numerous instances the malignant cells were killed when there was no visible effect upon the normal.

These figures include results obtained with tube cultures of the rat tumors and of mouse C<sub>57</sub> No. 350. Rat tumor 120 was less affected than the others, but the selective effect of penicillin upon the malignant cells of this tumor was unquestionable. The behavior of the mouse tumor B.A. No. 37, on the contrary, proved to be so like that of normal mouse tissue in its reaction to penicillin that the presence of a selective effect was doubtful. In three tubes there were 10 muscle colonies showing incipient effect, 16 showing marked

TABLE I  
*Number of Explants of Sarcoma Cells and of Normal Fibroblasts Showing  
Different Grades of Damage. Combined Totals of All Experiments*

	None	Incipient	Marked	Lethal	Total
Colonies of normal tissue.....	112	92	57	0	261
Sarcoma.....	0	29	208	104	341

damage, and 4 dead, as against the cultures of No. 37 which showed 18 markedly damaged colonies and 7 dead. A dose heavy enough to kill the malignant cells had also killed some of the normal, and the slight advantage of the normal is of questionable significance.

The time at which damage appeared varied with the different cultures and doses, but typically, using a dose at the selective lethal level, an incipient damage to tumor cells could be detected at 12 hours. There was marked damage at 24 hours, and complete killing of the growth zone at 48 hours. By 48 hours, however, there was sometimes a new growth of tumor cells, already pushing out from the explants. If this reviving growth was then given fresh medium free from penicillin, the tumor cells grew vigorously. If the fresh medium contained penicillin, however, this new growth was in turn killed off. Four to six days (*i.e.* two to three changes of penicillin medium) were usually sufficient to eliminate all tumor cells from explants 2 mm. in diameter. Then when the medium free from penicillin was added no tumor cells grew out, or if the sarcoma explants were implanted into rats, no tumors formed. With explants of 1 mm. diameter, however, 2 days sufficed to kill the malignant cells, as determined microscopically and by implantation.

The stroma included in the explants of the various tumors responded much the same as did the fibroblasts growing from the muscle fragments, but was perhaps slightly more susceptible than the normal, and grew more slowly than either muscle fibroblasts or malignant cells. In some tube cultures it was possible, without killing the normal cells, to kill all cells in the tumor explants (rat tumors 104, 132, 304, and mouse tumors C<sub>57</sub> 350), but there may not have been any stroma included in these explants. More frequently, apparently dead explants (initial migration zone disintegrating and no new cells migrating from the explants during treatment) recovered enough in a medium free from penicillin to send forth at least a few stromal cells. These long, fusiform cells resembled fibroblasts rather than the stout, multipolar malignant cells.

As a final safeguard against classifying viable malignant explants as killed, those pieces which showed no growth and those which showed a growth of stroma or of some doubtfully malignant cells (Figs. 2 and 5) were implanted in young rodents of the corresponding inbred strain. The failure of these cells to grow into tumors showed that the estimation of lethal effect had been conservative in that some malignant explants graded as merely damaged failed to produce tumors. Colonies in four tubes graded as 100 per cent lethal, failed to produce tumors when implanted into animals; and out of 21 tubes graded as probably containing surviving tumor cells, only six contained tissue capable of producing tumors. It is worthy of note that only a few untreated cells are necessary to produce a tumor *in vivo*. To verify the susceptibility of the animals, they were given implants of untreated as well as the penicillin-treated tumor tissue.

In two cultures the dosage in Oxford units was determined by bacteriological assay of the penicillin solution.<sup>2</sup> Rat tumor 304 was killed at a level of 59 units per cc. of Reichel lot 1C533. The muscle fibroblasts in these cultures showed marked damage. The malignant cells of rat tumor 132 were markedly damaged (without any damage to the normal fibroblasts) by 75 units per cc. of Squibb control 87225 and by 73 units per cc. of Squibb control 91478.

#### DISCUSSION

The evidence is fairly conclusive in showing that the agent producing the selective damage was in the penicillin preparations. The effect increased with the increase in dosage, and in control tubes identical except for the lack of penicillin, the malignant cells grew at least as well as the fibroblasts.

The revival of growth from the tumor explants after 2 days treatment suggests the possibility of a synergistic effect of penicillin along with some com-

\* We are indebted to the Bacteriological Division of the Food and Drug Administration for the assays.

ponent of the medium. The hydrogen ion concentration is one such possibility, since fresh medium is more alkaline than medium in which tissues have been growing. However the pH alone is not responsible for the selective lethal effect since indicator dyes showed no difference between treated and control tubes, and no changes upon addition of penicillin. More probably the recovery of the malignant tissue resulted from breakdown of the penicillin. This possibility was checked by transferring the penicillin medium from one tube, in which it had killed tumor cells, to an untreated culture.<sup>3</sup> The malignant cells in the second culture were uninjured, clearly indicating that the medium had lost its potency.

Consideration must be given to the possibility that the medium favors the growth of the cells derived from the muscle, and that penicillin acts by merely lowering the life-supporting powers of the medium, whereupon the sarcoma succumbs first. The sustained superiority of growth of the malignant cells over the normal, however, indicates that the medium was entirely adequate. Omitting plasma or adding embryo extract during the penicillin treatment did not eliminate the selective lethal effect. Tests with different media may prove fruitful, however, in revealing whether penicillin acts upon an intrinsic peculiarity of malignant cells or merely upon a susceptibility created *in vitro*.

Although malignant growths have well established peculiarities of metabolism, few substances have been demonstrated to have a selective effect upon neoplastic cells *in vitro*. Chambers, Cameron, and Kopac (1) have reported injury to malignant lymphoid cells by three phenylenediamines. N,N,N',-N'-tetramethyl-*o*-phenylenediamine was toxic to leukemic cells at concentrations one-seventh that which affected normal lymphoid cells.

Bacteriostatic action apparently does not necessarily carry with it a sarcoma-damaging activity. The sulfa drugs, for example, are even used routinely in cultures of neoplastic tissue to reduce infection, and no selective effect has been reported. Indeed, we have no proof that the results reported here are effects of the bacteriostatic agent in the mixture rather than of some substance not eliminated during the purification of the penicillin preparations used in these studies.<sup>4</sup>

#### SUMMARY

An agent present in pharmaceutical Squibb and in Reichel penicillin preparations was found to exert a selective lethal effect upon rat and mouse sarcoma cells growing with normal cells in tissue cultures.

<sup>3</sup> M. R. Lewis, unpublished observations.

<sup>4</sup> Subsequent studies by Dr. M. R. Lewis have shown that the selective effect is not exerted by highly purified colorless penicillin, but rather that the effect is due to some substance present in the less highly purified samples along with the bacteriostatic factor (*Science*, 1944, 100, 314).

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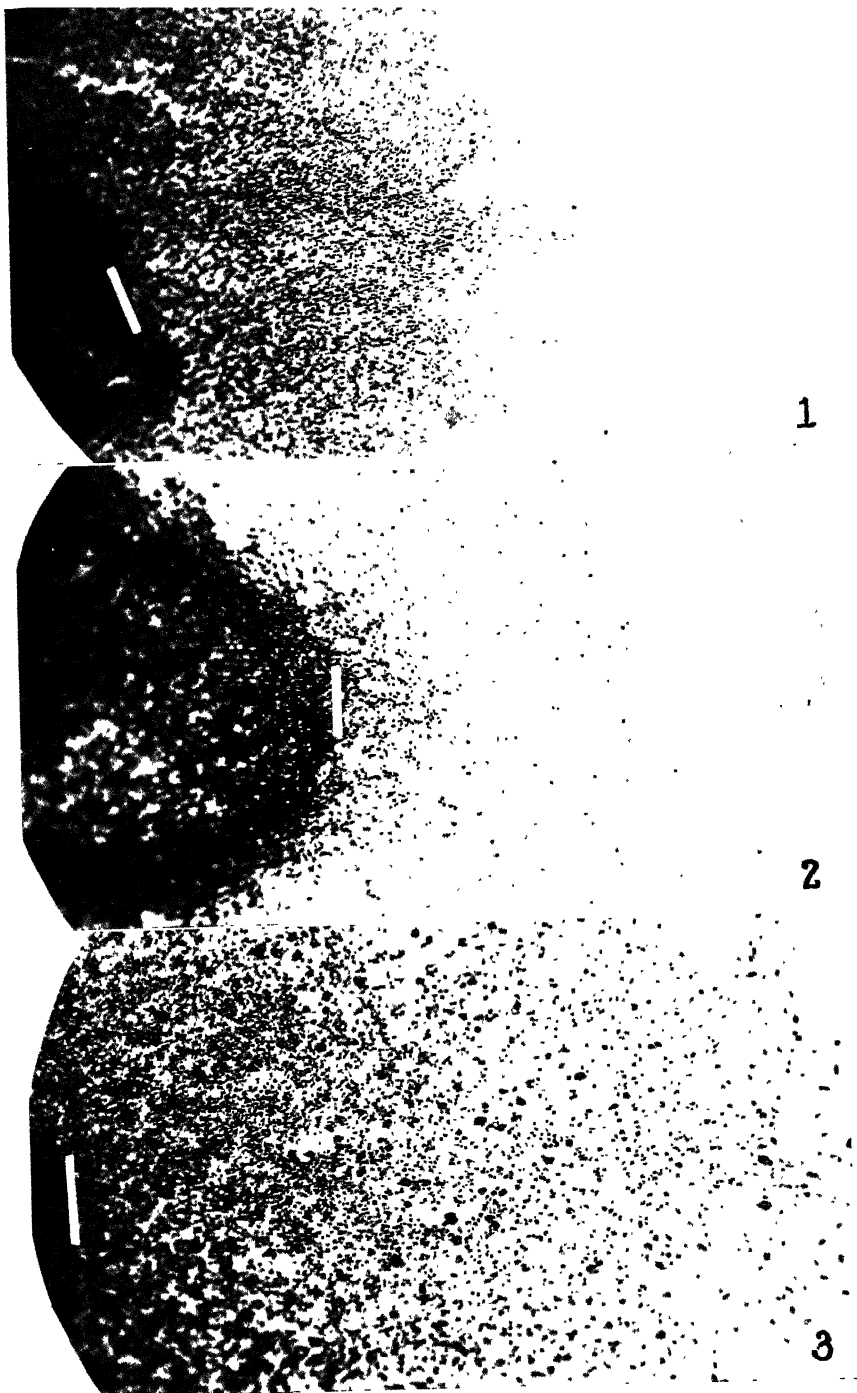
## EXPLANATION OF PLATES

## PLATE 1

The photomicrographs were prepared at the Army Medical School.

FIGS. 1 and 2. Muscle (Fig. 1) and sarcoma 132 (Fig. 2) growing in the same tube after 12 days' exposure to penicillin. The vigorous growth of the muscle forms a migration zone as broad as the diameter of the original explant, whereas the sarcoma shows only a sparse fringe of cells and scattered, rounded, moribund cells. A white bar indicates the edge of the explant.  $\times 45$ .

FIG. 3. Sarcoma 132, untreated. The 5 days growth is equal to the 12 days' growth of the muscle in Fig. 1.  $\times 45$ .



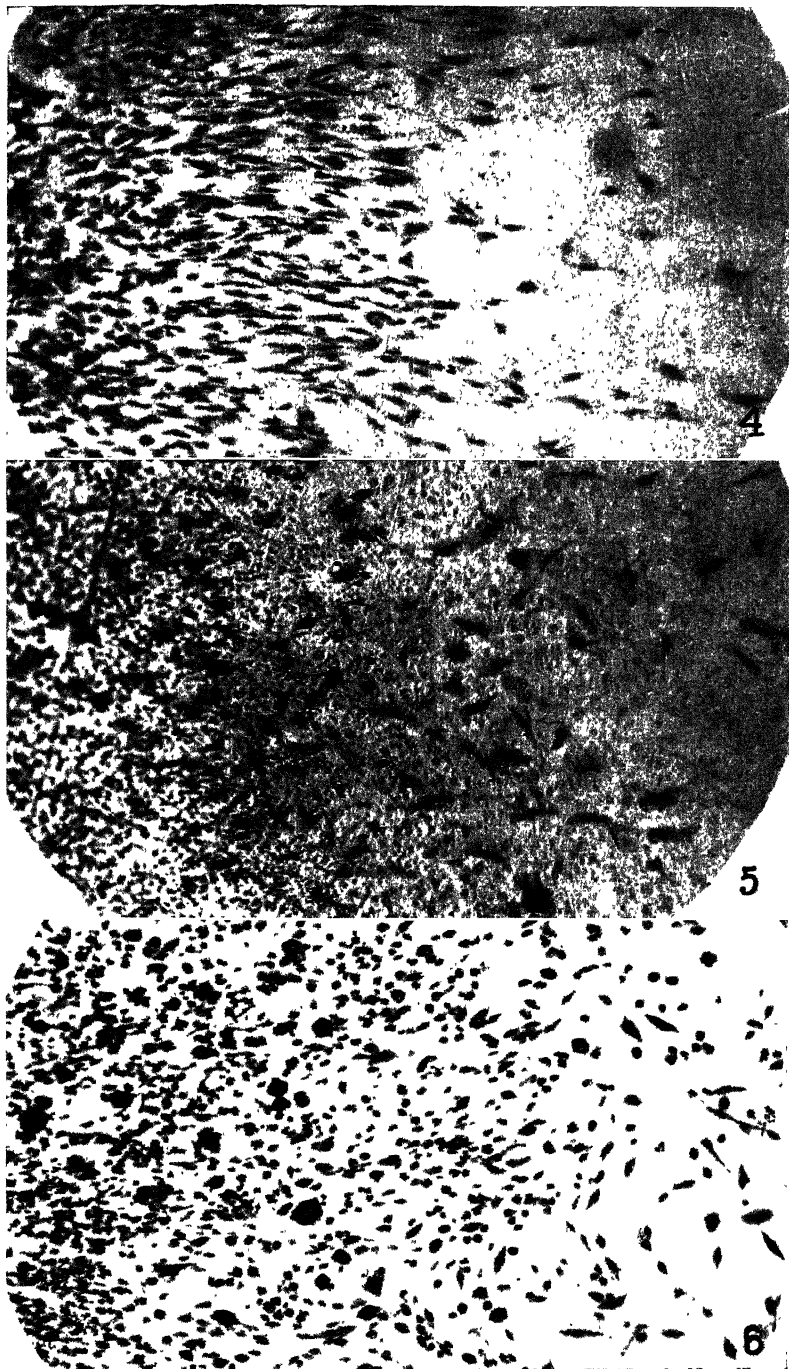
(Cornman: Selective lethal effect of penicillin on sarcoma cells)

## PLATE 2

FIGS. 4 and 5. Muscle (Fig. 3) and sarcoma 11 (Fig. 4) growing in the same tube after 6 days' exposure to penicillin followed by 2 days in normal medium. The cells in the muscle migration zone are the normal fibroblastic type whereas the sarcoma migration zone is composed only of deformed cells and debris of disintegrated cells. The effect was graded only as "marked damage," inasmuch as some apparently viable cells remain, but five sister colonies from the same tube, implanted into one rat, failed to produce a tumor.  $\times 100$ .

FIG. 6. Untreated cells of tumor 132. Same explant as in Fig. 3.  $\times 100$ .

Fixation in Bouin's; stained with hematoxylin.



(Cornman: Selective lethal effect of penicillin on sarcoma cells)





# THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

## XI. THE PREPARATION AND PROPERTIES OF "MEGAPERMSSELECTIVE" COLLODION MEMBRANES COMBINING EXTREME IONIC SELECTIVITY WITH HIGH PERMEABILITY

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### I

The classical investigations of Michaelis and collaborators<sup>1</sup> on the dried collodion membrane as well as the work of other investigators have revealed the basic physicochemical properties of membranes of porous character which are selectively cation-permeable.

Dried collodion membranes prepared from suitable collodion<sup>2-4</sup> were found to allow the cations of uni-univalent strong inorganic electrolytes in solution to pass through, whereas these membranes are almost impermeable to anions. This ionic selectivity has been shown to be due to the negative electrical charge of the membranes arising from the presence of dissociable acidic groups on the pore walls.<sup>4-6</sup>

The further study of the basic physicochemical properties of these membranes is impeded by certain unfavorable features, and their use in model systems is still more restricted for the same reasons. The electromotive properties of many of these membranes except in very dilute solutions do not approach the theoretically possible maximum values as closely as would be desirable; moreover they deteriorate during prolonged experiments. Their absolute permeability for non-electrolytes is very small, their ohmic resistance is very

<sup>1</sup> Michaelis, L., and Fujita, A., *Biochem. Z.*, Berlin, 1925, **158**, 28; 1925, **161**, 47; 1925, **164**, 23. Michaelis, L., and Dokan, S., *Biochem. Z.*, Berlin, 1925, **162**, 258. Michaelis, L., and Hayashi, K., *Biochem. Z.*, Berlin, 1926, **173**, 411. Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1926-27, **10**, 575. Michaelis, L., McEllsworth, R., and Weech, A. A., *J. Gen. Physiol.*, 1926-27, **10**, 671. Michaelis, L., Weech, A. A., and Yamatori, A., *J. Gen. Physiol.*, 1926-27, **10**, 685. Michaelis, L., *Bull. Nat. Research Council*, No. 69, 1929, 119; *Kolloid-Z.*, 1933, **62**, 2, and other publications.

<sup>2</sup> Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1927, **10**, 575.

<sup>3</sup> Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **24**, 467.

<sup>4</sup> Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **25**, 7.

<sup>5</sup> Sollner, K., and Carr, C. W., *J. Gen. Physiol.*, 1944, **28**, 1.

<sup>6</sup> Sollner, K., and Anderman, J., *J. Gen. Physiol.*, 1944, **27**, 433.

high, and correspondingly the rate of exchange of the permeable ions through such membranes is extremely low. The absolute permeability of these membranes is indeed so small that in many cases weeks must elapse before quantities have penetrated which can be analyzed by microchemical methods.<sup>2,7,8</sup>

Some of these difficulties have recently been overcome to a certain extent. The "activated" collodion membranes prepared recently<sup>4,5</sup> have satisfactory electromotive characteristics which are maintained for prolonged periods. Their absolute permeability, however, is still extremely low. We have therefore undertaken to prepare membranes more suitable for further extensive experimentation.

The desired properties of such membranes can be enumerated briefly, as follows: (a) the membranes should show extreme ionic selectivity, even in solutions of relatively high electrolyte concentration; (b) the absolute permeability of the membranes for the permeable, "non-restricted" ions should be high; *i.e.*, their ohmic resistance should be low; (c) the membranes should not deteriorate to a significant extent even on prolonged contact with electrolyte solutions; (d) the membranes should be mechanically satisfactory, *i.e.* they should be uniform and strong enough to stand considerable handling and also should have a well defined shape, free from kinks and wrinkles.

In addition, the preparation of these membranes should be easy and reproducible.

## II

As indicated above the main experimental problem was to increase the absolute permeability of "activated"<sup>4</sup> collodion membranes without impairing their ionic selectivity.

In the first attempt to do this, dried membranes were prepared from oxidized collodion<sup>4</sup> and then swelled in various concentrations of ethyl alcohol. The permeability of these membranes increased markedly, but even when the collodion was swelled only a few per cent the characteristic concentration potential which originally had been 53 to 54 mv. dropped below 50 mv. Swelling of dried "oxidized membranes"<sup>4</sup> in alcohol and in alcohol vapor as well as the oxidation of alcohol-swelled membranes were likewise unsatisfactory.

A satisfactory solution of this problem consists in drying porous membranes oxidized with NaOH over a mandrel.<sup>9</sup>

The preparation of the new type of membranes can be carried out with several variations. The nature and concentration of the collodion solution used,

<sup>7</sup> Weech, A. A., and Michaelis, L., *J. Gen. Physiol.*, 1928, **12**, 55; see also Green, A. A., Weech, A. A., and Michaelis, L., *J. Gen. Physiol.*, 1929, **12**, 473.

<sup>8</sup> Netter, H., *Arch. Ges. Physiol.*, 1928, **220**, 107.

<sup>9</sup> We should like to thank Mr. Harry P. Gregor for valuable help in the preliminary stages of this work.

the method of preparation of the porous membrane, the method and the time of oxidation are the main variables. By suitably changing the conditions one can arrive at various degrees of ionic selectivity and permeability.

On account of the high absolute permeability and very high ionic selectivity shown by these new type membranes we suggest for them the designation "*megapermselective*" or "*permselective*" collodion membranes.

In the following paragraphs a description is given of the procedure that has proved to be the most satisfactory in the preparation of such membranes.

The preparation of these membranes at present requires some skill and patience, particularly if membranes of very low resistance are required. There is little doubt that further experience will lead to the elimination of some of the remaining difficulties, most likely by a more rigorous control of the conditions under which the membranes are prepared.

In order to obtain uniform and reproducible megapermselective membranes the porous membranes were prepared by pouring collodion solution on the outside of test tubes rotating horizontally.<sup>10</sup>

A small variable speed motor was used to drive five pulleys in series. On the axle of each pulley a rubber stopper was attached; 25 × 100 mm. pyrex test tubes each having a small hole in the bottom were fitted in a well centered position on the stoppers. The holes of the tubes had been sealed smoothly by allowing a drop of a concentrated sugar solution to dry in the hole while the tubes stood in an oven. (The sugar dissolves when the tubes are later placed into water; this allows removal of the membranes without damage from the casting tubes.)

A 4 per cent solution of collodion cotton (Baker collodion cotton, U.S.P., Pyroxilin) in absolute ether-alcohol (50:50) was poured slowly from a narrow-mouth bottle over the tubes while they were rotated, the drippings being caught in a beaker. The optimum speed of rotation was 15 to 18 R.P.M. If the speed is too great, the drops of collodion that form on the under side of the tube do not drop off but form ridges on the membranes; if the rotation is too slow, uneven spreading of the solution results. The rotating tubes must be covered evenly with collodion, for if any spots are missed, they cannot be patched without causing bumps and ridges. After the tubes had been rotated for 3 minutes, another layer was added in the same manner, and 3 minutes later a third and final layer was put on. The tubes were rotated for 8 more minutes; they finally were taken off the stoppers and immersed in distilled water which was changed repeatedly. After 30 minutes the membranes were ready for oxidation.

The porous membranes thus prepared were oxidized by placing the membranes still on the test tubes in 1 M NaOH for measured lengths of time.<sup>4</sup> After superficial washing with water they were then immersed in repeatedly changed distilled water and allowed to stand for 2 to 3 hours. The membranes still on the tubes were then taken out of the water and dried in air for several hours while standing in an upright position. Under most conditions a drying time of 5 or 6 hours gives satisfactory re-

<sup>10</sup> See, e.g., Bigelow, S. L., and Gemberling, A., *J. Am. Chem. Soc.*, 1907, **29**, 1576. Bigelow, S. L., *J. Am. Chem. Soc.*, 1907, **29**, 1675.

sults; with low relative humidities of the air ( $< 30$  per cent) the drying should be broken off as soon as some out of a batch of membranes begin to crack. While drying, the membranes are prevented by the glass tubes from shrinking in area as would occur without this rigid support. They thus undergo what amounts to a stretching in two dimensions, the whole volume loss on drying being compensated for by a decrease in thickness. To remove these dried membranes from the tubes, they were soaked in water for 1 to 2 hours. This soaking in water not only swells the membranes slightly<sup>11</sup> and makes them less brittle, but also provides a lubricant for their movement over the glass. For easy handling the glass-clear, perfectly smooth membranes were tied securely with linen thread to glass rings which just fitted inside the open end of the membrane bag. For further use they are kept in water to which a crystal of thymol has been added as preservative. The thickness of these membranes is about  $30 \mu$ .<sup>12</sup>

We may add that the use of NaOBr as oxidizing agent is not advisable for the preparation of megapermselective membranes; membranes thus prepared are not much different in their permeability properties from the previously described<sup>3</sup> types of "activated" membranes.

Two sets of measurements were made to characterize the membranes. Measurements of the "characteristic concentration potential" ( $0.1 \text{ M KCl}/0.01 \text{ M KCl}$ ) were made as a criterion of the ionic selectivity of the membranes, as described by Michaelis and collaborators.<sup>1</sup> For a measure of the absolute permeability of the membranes, their ohmic resistances were determined in  $0.1 \text{ N KCl}$  solution, using the Kohlrausch bridge method with an alternating current of about 1000 cycles per second. The measurements were made after 30 minutes' contact of the membranes with the KCl solution, though the resistance of some of the membranes still drops on longer immersion in the electrolyte solution.

<sup>11</sup> Carr, C. W., and Sollner, K., *J. Gen. Physiol.*, 1943, 27, 77.

<sup>12</sup> In preparing the membranes the concentration of collodion and drying times can be changed considerably without materially affecting the final results. The concentration of collodion should be such that it drains off the rotating tube without leaving any lumps. This will vary with the brand of collodion, as the viscosity of collodion solutions greatly varies with different preparations. The drying time between the casting of the three layers and the final drying time should be adjusted so that when the membrane is placed in water it remains clear and no white spots appear. The temperature has some effect, though not much, on the optimum drying times. It is, however, important that the temperature of the room in which the membranes are cast be below  $22^{\circ}\text{C}$ ., for if it is higher, bubbles are formed in the membranes. The number of layers should be such that the thickness of the final dried membranes is 30 to  $50 \mu$ . If they are appreciably thinner, they become very weak after the oxidation, and many of the membranes break while drying on the tubes or while they are being removed from the tubes. Membranes thicker than  $50 \mu$  do not seem to offer any advantages; they have, however, a lower permeability.

To determine the resistance of the bag-shaped membranes it was necessary to use special electrodes constructed and placed so that the current would pass in about equal density through the whole area of the membrane. The outside electrode consisted of a hexagonal cage 45 mm. wide and 120 mm. high made from thin glass rod and wound with platinum wire. The inside electrode was a platinum wire spiral wound round a glass rod. Both electrodes were platinized by electrolysis in 2 per cent chloroplatinic acid. To make a measurement a membrane was filled with 0.1 N KCl and clamped in position inside the cage electrode, which was immersed in a beaker filled with the same solution. The other electrode was then lowered to a fixed position into the inside of the membrane. The total resistance between the two electrodes was then measured. Next, the resistance of the system without the membrane was determined. Neglecting the resistance of the layer of solution replaced by the membrane, the difference in these two measurements can be taken as the resistance of the membrane.<sup>13</sup> Since all of the membranes were of approximately the same effective area (50 sq. cm.), the results of the resistance measurements are given below in the tables in ohms per membrane, as obtained experimentally.

As was pointed out previously,<sup>4</sup> oxidation with NaOH not only activates but also weakens porous membranes and finally destroys them. Therefore the effect of the time of oxidation in NaOH was studied (Table I).

In Table II are shown the results obtained with a number of membranes prepared with a uniform time of oxidation (but without rigid temperature control during the latter). The first eight membranes listed in this table were taken from eight different series to show the variation in properties which under these conditions may occur from one experiment to the next. The last five membranes in the table were prepared at the same time; they show the uniformity of the characteristics of the individual membrane specimens in one series.

### III

Table I shows that the "characteristic concentration potential" in all cases, with short as well as with long oxidation times, approaches the theoretically possible maximum of 55.1 mv. within 1 mv.; the resistance of the membranes, however, decreases sharply with increasing time of oxidation. One is able to produce at will membranes having resistances of almost any desired magnitude. Excessively strong oxidation obviously not only increases the

<sup>13</sup> In a few instances the resistance was also measured by using a direct current method. A constant E.M.F. caused a small current (*ca.* 10  $\mu$  amps.) to flow through a membrane and the electrode system. Then the membrane was removed from the system, a decade resistance box was added in series, and its resistance adjusted until the same current flowed as before. The resistance that had to be added corresponds to the resistance of the membrane. On account of membrane and electrode polarization, this method was only accurate to about 20 per cent. The direct current measurements agreed within this limit with the alternating current determinations.

number of pathways through the membrane, as indicated by the low resistance of the membranes, but also brings about an increase in the size of some of the

TABLE I  
*The Effect of the Time of Oxidation on the Properties of "Megapermselective" Collodion Membranes*

Oxidation time in 1 N NaOH	Characteristic concentration potential 0.1 M KCl/0.01 M KCl	Resistance in 0.1 M KCl ( $\pm 0.5 \Omega$ )	Water content in volume per cent
min.	mv.	$\Omega/50 \text{ cm.}^2$	
4	54.8	80	—
6	54.6	22	—
8	54.8	17	16.4
10	54.5	8	16.3
12	54.5	3	15.9
14	54.6	2	—
16	54.0	1.5	18.0
18	Membrane destroyed		

TABLE II  
*Characteristic Concentration Potential and Resistance of "Megapermselective" Collodion Membranes*

Membrane No.	Characteristic concentration potential 0.1 M KCl/0.01 M KCl ( $\pm 0.1 \text{ mv.}$ )	Resistance in 0.1 M KCl ( $\pm 0.5 \Omega$ )
	mv.	$\Omega/50 \text{ cm.}^2$
a (3)	54.5	11.0
b (4)	54.5	10.5
c (1)	54.6	2.5
d (1)	54.2	3.0
e (5)	54.8	6.0
f (1)	55.1	$1.0 \pm 0.2$
g (1)	55.0	$0.5 \pm 0.2$
h (1)	54.9	$0.5 \pm 0.2$
i (1)	54.5	3.0
i (2)	54.8	3.0
i (3)	54.7	3.0
i (4)	55.1	3.0
i (5)	54.7	3.0

pores so that they permit the passage of some anions; the membranes become slightly leaky and the concentration potential is therefore slightly lowered.<sup>14</sup> It

<sup>14</sup> The "leak" of anions can be calculated from the potential measurements. If  $\bar{u}$  and  $\bar{v}$  are the relative contributions of the cations and anions to the transport of current

should also be mentioned that the strength of the membranes decreases with increase in oxidation time. Membranes with resistances above 50 ohms can stand considerable handling without breaking, while the membranes of less than 10 ohms are fairly weak and break rather easily if not treated with care. The water content of the membranes varies from 16 to 18 volume per cent. This compares with a water content of about 10.5 volume per cent of conventional dried membranes prepared from the same collodion, this value being the same both for the original commercial and oxidized ("activated") preparations.<sup>11</sup> The higher water content, the great porosity, of the mega-

TABLE III  
*The Influence of Shrinking on the Properties of "Megapermselective" Collodion Membranes*

Membrane No.	Megapermselective membranes		The same membranes in the shrunken state	
	Characteristic concentration potential 0.1 M KCl/0.01 M KCl	Resistance in 0.1 M KCl	Characteristic concentration potential 0.1 M KCl/0.01 M KCl	Resistance in 0.1 M KCl
	<i>mv.</i>	$\Omega/50 \text{ cm.}^2$	<i>mv.</i>	$\Omega/50 \text{ cm.}^2$
j (2)	55.0	90	55.2	700
k (4)	55.1	30	54.9	90
l (3)	54.6	13	54.0	200
m (4)	54.7	15	54.0	175
n (1)	54.2	3.0	54.5	77

permselective membranes must be assumed to be one of the most important causes of their great absolute permeability.

Table II shows the still prevailing variation between different series and the uniformity of the different membrane specimens in any given series. The membranes keep their properties for several weeks on contact with water without any significant deterioration. Their ionic selectivity slightly decreases on prolonged contact with electrolyte solutions.

in the membrane, we may use the Nernst equation for the diffusion potential in this form:

$$E = \frac{\bar{u} - \bar{v}}{\bar{u} + \bar{v}} RT \ln \frac{c_1}{c_2}$$

The numerical value of  $RT \ln \frac{c_1}{c_2}$  in the case of 0.1 N KCl/0.01 N KCl, correcting for known activity coefficients, is 55.1 mv. If, e.g.; a potential of  $E = 54$  mv. is found with a membrane under these conditions the "leak" of anions can be calculated from the above equation. Introducing the numerical values in this equation we obtain the ratio:

$$\frac{\bar{u}}{\bar{v}} = \frac{109.1}{1.1},$$

corresponding to an anion leak of about 1 per cent.



The conventional dried collodion membranes do not change on repeated drying and wetting; they can be stored for months in the dry state without any changes in properties. Since the megapermselective membranes are dried over a form and thus are in a forced, stretched state, it was necessary to investigate whether or not they shrink if dried without support in the air and thereby change their properties. Some shrinkage did occur when the membranes were dried in air after they were removed from the tubes. The shrinkage was just visible, the membranes became very slightly wavy. The effect of this shrinkage on the concentration potentials and resistances of several membranes is shown in Table III.

Table III shows that drying in air without support greatly increases the resistance of the megapermselective membranes. The electromotive properties, however, which before shrinking were already very near the maximum, do not change significantly. The use of "shrunk" membranes will be profitable only in those instances in which dry storage of the membranes is desirable.

#### IV

To demonstrate the great absolute permeability of the megapermselective membranes in the most direct manner we have performed a few preliminary experiments on cation exchange across them. In one such experiment a bag-shaped membrane ( $1\Omega$  resistance in  $0.1\text{ N KCl}$ ) with an active area of about  $50\text{ cm.}^2$  containing  $30\text{ ml.}$  of  $0.1\text{ N NH}_4\text{Cl}$  solution was placed in a tube containing  $30\text{ ml.}$  of  $0.1\text{ N KNO}_3$  solution. After 4 hours without stirring the  $\text{KNO}_3$  solution had become  $0.03\text{ N}$  in  $\text{NH}_4^+$  ion concentration. This represented an exchange of  $0.9\text{ m.eq.}$  of cations during this period, while less than  $0.02\text{ m.eq.}$  of chloride ion "leaked" out into the  $\text{KNO}_3$  solution, the anion leak in this experiment being about 2 per cent. The leak through membranes of somewhat lower absolute permeability ( $3$  to  $5\Omega$  in  $0.1\text{ N KCl}$ ) under the same conditions is much smaller, ordinarily below 1 per cent. Stirring increases the absolute rate of ion exchange several fold. The relative leak in more dilute solutions is always less, the leak of bivalent anions in similar experiments being immeasurably low. Our results indicate, in agreement with expectation, that there is a fair parallelism between the conductance of these membranes and their absolute permeability as measured by exchange rates.

The rate of cation exchange through megapermselective membranes is about two to four orders of magnitude greater than the rate reported by previous investigators,<sup>2,8</sup> moreover, the leak of anion is smaller—the ionic selectivity of the new membranes being much greater.

We have also tested the water permeability of the megapermselective membranes. A solution of  $0.2\text{ M}$  sucrose was placed inside of a membrane of  $3\Omega$

# CHANGES IN THE APPARENT CYTOPLASMIC HYDROGEN ION CONCENTRATION OF AMEBA DUBIA ON INJECTION OF EGG ALBUMIN

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## INTRODUCTION

At the time this work was done more than ten years ago, no satisfactory explanation of the results could be found. The observations required that protein introduced into the medium containing the amebae, rapidly enter these cells, and this was considered unlikely. Recent work with entirely different materials and methods has indicated that this is not at all improbable (7). The results are therefore being presented with an interpretation based on the assumption that the protein used does enter the cell. The experiments to be described furnish evidence of the presence of a bicarbonate buffer system in the cytoplasm and show that the nucleus is functionally associated with the maintenance of the intracellular pH.

The early work of Chambers and his coworkers (4-6, 9-11) has been interpreted as indicating that the cytoplasm of various kinds of cells is uniformly at pH  $6.9 \pm 0.1$ . This pH was maintained as long as the cell remained alive even in the presence of acids or alkalies in the surrounding medium. They explained their observations by assuming that the cytoplasm is a buffered system and as such tends to maintain a uniform characteristic pH. Ammonia and carbon dioxide were striking exceptions to the behavior of other reagents in that they made the apparent pH of the cytoplasm respectively more alkaline or more acid.

More recently Spek (14) and Spek and Chambers (15) have described two components of the cytoplasm which give different staining reactions, the hyaloplasm which shows the color of the dye at about pH 7.6, and granuloplasm which has a more acid-staining reaction at about pH 5.0.

## *Materials and Methods*

Egg albumin was crystallized with ammonium sulfate and sulfuric acid by the method of Sørensen (12) and then three times recrystallized. The crystals were dialyzed against distilled water for 48 hours by the method of Abramson and Grossman (1, 2) and then dried under reduced pressure over sulfuric acid. A 2 per cent solution of this dried product in glass-distilled water was prepared and kept at 2 to 6°C. Small samples were colored with a few drops of phenol red before injection. The pH of the injected solution was about 5.0.

All injections were made with a Chambers' micromanipulator, using a  $43\times$  objective and  $10\times$  ocular. pH determinations were made by comparing the color of the cytoplasm under a  $10\times$  objective and  $10\times$  ocular with a set of Clark standard tubes. The light source was a ribbon filament lamp with a white opal glass screen. Pipettes for handling amebae were made of pyrex glass, as were the micropipettes used for injection. All the amebae used were grown in Pace's medium (8). While making the injections the amebae were mounted in Pace's medium (without the wheat) and in glass-distilled water. The phenol red solutions used were made up with 28.2 cc. of 0.01 N NaOH per 0.1 gm. of the dye and diluted to 25 cc. with distilled water to give the 0.4 per cent reagent. The descriptions to follow, unless otherwise indicated, will be summaries of individual protocols.

#### OBSERVATIONS

##### *A. Changes in the Streaming and Configuration of the Cytoplasm*

*I. Amebae Mounted in Pace's Solution and Injected with 2 Per Cent Albumin Colored with Phenol Red.*—Immediately after injection of amounts varying from two to six or seven times the volume of the nucleus, the plasmalemma is raised to form one or two hyaline "blisters" equal in volume to one-half or the whole of the ameba. The hyaline area rapidly spreads to include the entire periphery of the ameba. Rotary streaming of the hyaline and granular cytoplasm, as described by Chambers and Reznikoff (4) for distilled water injections, then sets in. The hyaline area then becomes reduced to approximately normal proportions, the animal becomes elongate and monopodial, with streaming of cytoplasm in a central column toward the anterior (see Figs. 1-3).

With repeated injections the broad hyaline and granular regions remain distinct for a longer time, streaming movements are less frequent. When streaming occurs it proceeds as follows: The hyaline region of the anterior end broadens (Fig. 4); the more finely granular cytoplasm then streams into this broadened area from one or both sides and occasionally through the middle (Fig. 5), but the border between the granular and hyaline cytoplasm remains distinct; this border is then suddenly swept posteriorly and becomes lost as a definite boundary in the posterior end of the animal (Fig. 6).

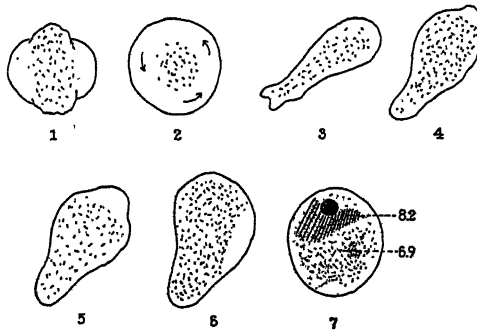
After the later injections (sixth, seventh) marked Brownian movement appears in the more hyaline regions and continues until the "boundary" between the heavily granular and more hyaline cytoplasm passes through them.

After about five injections there is also a noticeable change in the surface layer of the ameba. It seems to be much more easily penetrated by the pipette than in the first or second injections, and apparently adheres more firmly to the coverslip, since it is not as readily dislodged upon removal of the pipette as in the previous injections.

When the injections are made with the amebae mounted in distilled water the behavior is the same as described above. Such distilled water cannot

be considered free of salt, for when the animals are placed in a large volume of the same water or washed repeatedly with distilled water they become detached from the substrate, the pseudopodia become long and slender, and eventually all movement ceases. We must consider therefore that small volumes of distilled water dissolve enough salt from the container or the coverslip to maintain the osmotic balance.

*II. Amebae Mounted in Pace Solution and Injected with 0.4 Per cent Phenol Red.*—After injection of phenol red the plasmalemma is not raised as in the albumin injections and the animal retains its polypodial form. Sometimes there are one or two aggregations of crystals, apparently held together by



FIGS. 1 to 6. Successive changes in the form of an ameba injected with 2 per cent albumin in distilled water.

FIG. 7. An ameba which received one injection of phenol red followed by eight injections of 2 per cent albumin, 10 hours after the last injection. A portion of the cytoplasm has a color indicating a pH of about 8.2, while the rest of the cytoplasm is at pH 6.9.

a localized gelation of the cytoplasm. Streaming of the cytoplasm may be slow or may stop completely, but is resumed again at the usual rate. In the former case the normal rate is resumed in about 3 minutes, in the latter in half an hour. Coincident with this change is the shift back to the normal pH as described below. The first injection is very easily made, but the third and subsequent injections are increasingly more difficult. An interfacial boundary is formed about the fluid being injected. The droplet so formed is usually expelled from the cytoplasm. When the droplet is retained, it has a distinct boundary about it. These droplets lose their color completely in a few seconds. After a few minutes the droplets themselves disappear.

### *B. Color Changes*

The color observed immediately upon injection of 0.4 per cent phenol red is similar to that of a standard tube at pH 6.6 or 6.7. Within 5 seconds the

color shifts to that characteristic for pH 7.0 to 7.2. When normal streaming is resumed (the time will vary depending upon the extent of the apparent gelation induced by the injection) the color is shifted back to pH 6.9. This color appears uniform throughout the cytoplasm except in the posterior regions where there are minute spherules that are definitely pink; *i.e.*, at a pH of about 7.4 to 7.6

*I. Amebae Mounted in Pace's Medium and Injected First with a Solution Made up of Equal Parts 0.4 Per Cent Phenol Red and M/208 Calcium Chloride.<sup>1</sup> Succeeding Injections Were of 2 Per Cent Albumin Solution.*—The albumin injections were made 2 hours after the injections of the dye. Following a momentary shift to the acid range, there was a shift to the more alkaline color. (This preliminary acid shift was not always observed.) For example, at the second injection two amebae shifted from pH 6.9 to pH 7.1. Another ameba shifted from pH 7.1 to pH 7.4. Four minutes after the injection the nucleus was slowly extruded. When this enucleated protoplast was injected 3 minutes later there was a shift to the more acid pH 7.1 and then to the more alkaline again, pH 7.4 to 7.6.

With further injections of albumin into non-enucleated amebae, *i.e.* 3rd, 4th, etc., there are similar successive shifts with each injection; for example, from 7.0 to 7.4, 7.6 to 8.0, 8.0 to 8.4. In a total of eleven amebae injected in this manner, ten showed a shift to a more alkaline condition. The one ameba which did not, changed from 8.0 to 7.1. It then rounded up and became colorless and motionless in 15 minutes.

*II. Amebae Mounted in Pace's Solution and Injected with 2 Per Cent Albumin Colored with Phenol Red.*—The effect of the first few injections cannot be followed because the color of the cytoplasm is too faint. Later injections show the alkaline shifts similar to those described above. Six such cases were observed. One animal showed no alkaline shift. It ruptured suddenly after the fifth injection.

With animals mounted in distilled water and injected with albumin colored with phenol red the behavior is similar. Four showed alkaline shifts. Two showed apparent acid shifts but are not considered significant since these were only second injections and the color was rather faint.

### *C. Enucleated Protoplasts*

During the course of some violent injections the nucleus along with a considerable amount of cytoplasm was forced out of the cell. In one case, the membrane reformed about the enucleated protoplast which had a pH of about 7.2. No further observations were made on this animal. In other cases, however, the nucleus was slowly extruded through the plasma membrane

<sup>1</sup> The dye solution had been stored in a soft glass bottle. The calcium chloride was added in order to counteract any possible effect of Na<sup>+</sup> and K<sup>+</sup> dissolved from the glass.

some time after the injection was made. Four such cases were observed and are described below:

The ameba was mounted in distilled water and injected with 2 per cent albumin colored with phenol red. After the second injection, the cytoplasm showed a pH of 6.9. When pierced with the pipette there was a momentary acid shift, then a change to about 7.1. When punctured after the third injection, there was a change to about pH 7.3. Albumin was then discharged into the medium about the ameba and the cytoplasm took on a violet color (about pH 8.6). An hour and a half later the color was red (about pH 7.2 to pH 7.4). A volume of albumin about two times that of the nucleus was then injected. The color of the cytoplasm indicated a pH of 8.2 to 8.4. The plasma membrane then became crenulate and streaming of the cytoplasm set in. The nucleus was then slowly extruded through the plasma membrane. The enucleated protoplast then streamed away in an apparently normal manner with the cytoplasm at pH 8.2 to 8.4. When albumin was discharged about the enucleated ameba the pH shifted to 8.6. Another injection of albumin was then made, and the color became violet (8.6 or higher). When the animal was now pierced the color became bluer.

Another ameba mounted in the same medium extruded its nucleus after the second injection of albumin when its cytoplasm was at a pH of about 7.4. The enucleated protoplast was then injected and a shift to pH 7.5 or 7.6 was noticed. 12 hours later the pH was about 8.2 and the cytoplasm was still actively streaming.

A third ameba was first injected with phenol red and then given injections of albumin, colored with the same dye. After the third injection the pH shifted from 8.0 to 7.7 locally in the region of the injection and then to 8.2 throughout the cytoplasm. The nucleus was then slowly extruded. One hour later the cytoplasm was actively streaming and had a color comparable to pH 8.2 to 8.4. Three hours later streaming was still active with the cytoplasm at pH 8.6 or higher.

A fourth ameba was injected under conditions similar to the previous one. After the fourth injection the pH shifted from 7.6 to 8.3. When albumin was then discharged about the animal there was a shift to pH 8.6. The nucleus was then slowly extruded and the enucleated protoplast streamed away with the cytoplasm about pH 8.2. It then rounded up and the crystals aggregated at one end. There was no color change on piercing. Twelve hours later the animal was colorless and apparently dead.

It was noted that all the enucleated animals which remained alive showed more alkaline pH values on standing whereas those with the nuclei retained, either shifted back to pH 6.9 completely or, where the injections had been numerous and large, contained localized regions of about  $\frac{1}{4}$  to  $\frac{1}{3}$  the size of the animal with a pH of 8.2 or higher while the rest of the cytoplasm was at pH 6.9 (Fig.7).

#### *D. Gas Exchange*

The rapidity with which the color changes are produced by injection or discharge makes it seem likely that ionic interchanges rather than digestive processes are responsible for the observed behavior. If there are carbonates

and bicarbonates in the cytoplasm, the albumin introduced inside the cell may replace the bicarbonate associated with base resulting in the liberation of free  $\text{CO}_2$ . Since the color changes observed with phenol red are similar when the albumin is brought in contact with the cell surface to when it is injected one might expect  $\text{CO}_2$  to be given off when albumin is added to the medium containing the cells. To determine whether the bicarbonate in the cytoplasm might be involved in producing the change in the color reaction of the cell an experiment was performed, designed to measure changes in  $\text{O}_2$  and  $\text{CO}_2$  pressure when amebae were brought in contact with albumin.

It was found that by agitating cultures, and then allowing them to settle, the amebae soon became adherent to the bottom of the container and the debris along with foreign organisms could be removed by pouring off the culture medium. Fresh Pace solution (with no wheat or other organic material) was then added and the process repeated about ten times. The cultures were then allowed to stand overnight and the washings repeated on the following day. Examinations under the microscope showed the cultures to be free of all foreign material. The amebae were then concentrated (without centrifuging). A sample of the concentrated suspension was then centrifuged at 3300 R.P.M. for 45 minutes in a Hopkins vaccine tube. The concentration of amebae was found to be approximately 0.005 cc. per cc. of suspension. Three cc. of this suspension were then placed in the chamber of each of two small conical Warburg vessels, one of which had 0.1 cc. of 2 N KOH in the central well, the other 0.2 cc. Pace solution. Half a cc. of 2 per cent albumin in glass-distilled  $\text{H}_2\text{O}$  was placed in the side arm of these vessels. Control vessels contained (1) 0.4 cc. Pace solution in the chamber, 0.5 cc. 2 per cent albumin in the side arm, nothing in the well; (2) 3 cc. Pace solution, 0.5 cc. 2 per cent albumin in the side arm, and 0.1 cc. 2 N KOH in the well; (3) 6 cc. distilled  $\text{H}_2\text{O}$  in the chamber, no KOH, no albumin. Vessels 1 and 2 were used as barometers and checked against 3. They were tipped to bring the albumin into the chamber at the same time that the experimental vessels were. Readings were taken every 5 minutes for 2 hours, the vessels then tipped to bring the albumin into the chamber and readings continued for 2 more hours. Prior to the introduction of albumin, the amebae consumed  $14.4 \text{ mm.}^3$  of oxygen per hour, during the same period they produced  $17.0 \text{ mm.}^3 \text{ CO}_2$  per hour. After the albumin was introduced into the chamber they consumed  $17.1 \text{ mm.}^3 \text{ O}_2$  per hour and produced  $35.9 \text{ mm.}^3 \text{ CO}_2$  per hour.

Before the amebae were in contact with albumin they produced  $\text{CO}_2$  at a rate of  $1.4 \text{ mm.}^3$  per 5 minutes. In the 4 minutes immediately following tipping of the vessel  $9.7 \text{ mm.}^3 \text{ CO}_2$  was produced, in the next 5 minutes 8.5, then 3.7, 3.0,  $1.7 \text{ mm.}^3 \text{ CO}_2$ . The rate then became uniform as it had been prior to the introduction of albumin and averaged  $1.2 \text{ mm.}^3$  per 5 minutes. There was no rise in the rate of  $\text{O}_2$  consumption during the period when the  $\text{CO}_2$  evolution was very high. The slight increase for the 1 hour period in albumin ( $3 \text{ mm.}^3$ ) was due to a slightly high rate throughout the hour and not to a marked increase immediately after the introduction of albumin as in the case of  $\text{CO}_2$ . Rates per 5 minutes of  $\text{O}_2$  consumption after albumin were: 2.7, 0, 1.8, 0.9, 2.7,  $0.9 \text{ mm.}^3$ . Microscopic examination of the amebae at the end of the experiment showed them to be motile and apparently normal.

The results are shown graphically in Figs. 8-10. They may be taken to indicate that the bicarbonates of the cell are involved in the reaction, the free  $\text{CO}_2$  being produced by replacement of bicarbonate ions by the albumin.

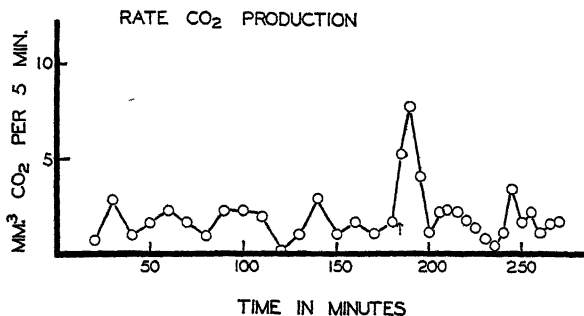


FIG. 8

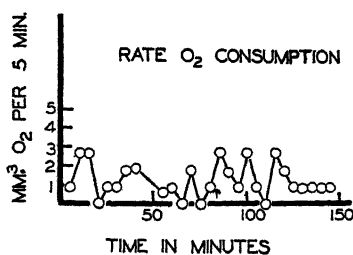


FIG. 9

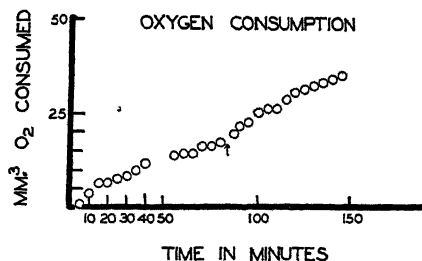


FIG. 10

FIGS. 8 to 10. Oxygen consumption and carbon dioxide production before and after contact with 2 per cent albumin. The arrow indicates the time the albumin was brought in contact with the amebae.

The more or less cyclic changes in the apparent rate of  $\text{O}_2$  consumption and  $\text{CO}_2$  production are probably due to inadequate shaking of the vessels. The shaking frequency was kept low (8 times per 10 seconds) to avoid injury to the cells.

#### DISCUSSION

The results observed cannot be explained solely on the basis of a Donnan membrane equilibrium. It is difficult to understand how evolution of  $\text{CO}_2$  can be produced by the albumin unless the latter actually enters the cells. The experiments with phenol red point to the same conclusion since the color changes are similar whether the albumin be injected or merely brought to the cell surface. However, this alone does not adequately explain the results since egg albumin added to a bicarbonate solution causes a drop in pH whereas an apparent rise was observed. It seems probable therefore that although both the dye and the albumin readily permeate the cytoplasm when injected they nevertheless become rapidly distributed to different phases of it. Sim-



ilarly, if the albumin enters the cell through the surface membrane it passes into a phase of the cytoplasm other than the one containing the phenol red. Evidence of the existence of at least two phases in the cytoplasm has been obtained by different methods and for various types of cells by Spek and Chambers (15), Baas-Becking and his coworkers (3), and by Seifriz (13). Apparently, albumin when it first enters the cell, displaces  $\text{HCO}_3^-$  but then becomes bound to a micellar surface and effectively removed from the medium containing the phenol red, resulting in a more alkaline coloration of the latter. When the nucleus is present enough  $\text{CO}_2$  is produced to bring the pH back to 6.9. In the absence of the nucleus, however, this process proceeds at too slow a pace to restore the original pH within 12 hours.

#### SUMMARY

1. Egg albumin when injected into an ameba or discharged into the solution about it raises the apparent pH of the cytoplasm of the ameba.

2. With time the cytoplasm returns to the original pH 6.9 if the nucleus is present. Amebae that have received repeated injections of albumin in some cases extrude their nuclei. In these cells the cytoplasm remains at the more alkaline pH induced by the albumin for at least 12 hours.

3. When a 2 per cent solution of albumin is introduced into a suspension of amebae there is a temporary marked rise in the rate at which  $\text{CO}_2$  is given off with no corresponding rise in  $\text{O}_2$  uptake.

4. The results observed can be explained if the albumin discharged onto the surface of the ameba rapidly enters the cell and there becomes distributed in a phase of the cytoplasm other than the one which contains the phenol red.

I wish to thank Professor Robert Chambers for his very helpful advice and for the use of a micromanipulator.

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# CULTURE CONDITIONS AND THE DEVELOPMENT OF THE PHOTOSYNTHETIC MECHANISM

## II. AN APPARATUS FOR THE CONTINUOUS CULTURE OF CHLORELLA

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It has been a common experience that the photosynthetic behavior of algal cells may vary within rather wide limits depending upon previous conditions of culture. However, no attempt has been made to relate culture conditions to the subsequent type of behavior of the cells. A very great handicap to any such investigation is the internal variation which occurs with time within any one culture. In the first paper of this series (Myers, 1944) it has been pointed out that as a culture of *Chlorella* matures, the  $H^+$  and  $NO_3^-$  ion concentrations of the medium, the conditions of carbon dioxide supply, and the effective light intensity and quality show marked variations. There are probably other variables as well; e.g., the "inhibitors" of Pratt (1942). Consideration of the nature of these internal variables shows that all of them are functions of the population.

It has been a common practice in studies on photosynthesis to use algal cells from cultures harvested at some fixed period after inoculation. By using cells taken always at the same point on the growth curve a fair degree of reproducibility is to be expected, since all conditions which depend upon the population will be reproduced with some uniformity. Similar results may be obtained by periodically harvesting a part of the culture suspension and replacing it with fresh medium. Techniques of this kind have been reported by Felton and Dougherty (1924) for the culture of pneumococcus and by Ketchum and Redfield (1938) for the culture of marine diatoms. It would seem that if a culture could be continuously diluted so as to be maintained always at one point on its growth curve, then the effects of changing internal conditions might be eliminated entirely. This would

\* The work of this paper was begun when one of us (J. M.) was supported by a National Research Council Fellowship at the Smithsonian Institution. Improvement in design of the apparatus and the accumulation of experimental data were done at The University of Texas under a grant from the University Research Institute. The authors are indebted to these institutions for support and assistance. Acknowledgement is made of the technical assistance of Mary Benjamin Smith.

afford at once (1) a source of experimental material of high uniformity and/or (2) a means of stabilizing internal variables so that relation of culture conditions to photosynthetic behavior might be systematically explored.

Following these considerations there has been developed an apparatus which maintains an algal<sup>1</sup> culture at a given density of population by automatically diluting the growing culture with fresh medium. This paper describes the apparatus and presents data illustrating its operation.

### *Description of the Apparatus*

Fig. 1 is a diagrammatic, cross-sectional view of the apparatus. A number of parts have been distorted in position in order to place all of them in the same plane. The culture chamber<sup>2</sup> is made of three concentric glass tubes, affording an outer annulus, *J*, for circulation of constant temperature water and an inner annulus, *A*, in which the algal suspension is contained. Outside dimensions of the chamber are approximately 6.0 cm. diameter by 66.0 cm. long; the only critical dimension is the thickness of annulus *A*, which in all chambers used is 5 to 6 mm.

Any desired gas mixture is provided through the bubbler tube, *B* (shown in part) which simultaneously provides carbon dioxide and agitates the algal suspension. (The gas mixture is first humidified by bubbling through a column of liquid medium held at the same temperature as the chamber.)

Because of the requirement of a rather rapid air flow (2 to 6 cc. per sec.) the use of compressed gas mixtures has proved uneconomical. It has been necessary to devise a means of obtaining constant air-carbon dioxide mixtures. Outside air is delivered *via* a diaphragm pump at a constant pressure obtained by allowing the excess air to escape against a head of 8 feet of water. Carbon dioxide is delivered from a cylinder at about 4 pounds pressure by suitable mechanical reducing valves. The two gases pass through orifices so chosen that the resulting gas mixture has a composition of about 4.4 per cent carbon dioxide. The actual rates of flow of the two gases are indicated by calibrated Venturi flow gauges and occasional Haldane analyses have been made as overall checks on the operation. The gas mixture is delivered at constant pressure by allowing the excess gas to escape against a head of about 6 feet of water.

Samples of the suspension are harvested as needed by opening a screw clamp and allowing the suspension to run out through the *withdrawal tube*. Fresh medium is added by activation of a solenoid valve, *SV*, which opens a rubber tube and allows the medium to run from a large aspirator bottle into the annulus *A* of the culture chamber.

By suitable precautions pure culture conditions can be maintained. The

<sup>1</sup> In principle it is applicable to other types of microorganisms as well.

<sup>2</sup> The first chamber was constructed by one of us (L. B. C.). Additional chambers have been obtained from E. Machlett and Son, New York City.

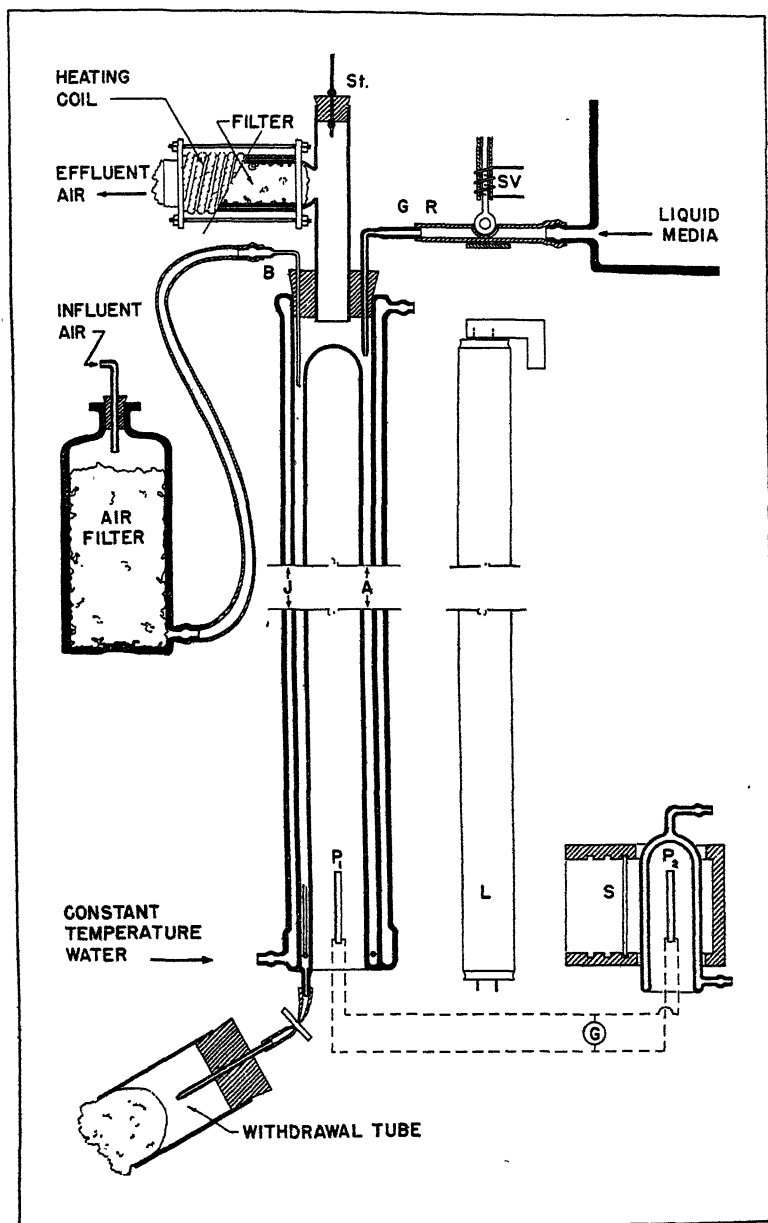


FIG. 1

hdrawal tube is protected by a glass skirt and cotton plug. After collection a sample in a sterile flask a fresh cotton plug, previously autoclaved

in a large test tube, is inserted in the glass skirt. Influent air is filtered through a 500 ml. aspirator bottle packed with cotton. Effluent air passes out through a glass tube packed with cotton. The cotton filters must be kept warm to insure against condensation of moisture. A heating coil around the effluent filter is shown partly cut away. A heater (not shown) for the influent filter is provided by a simple tin-can and light bulb arrangement. Before setting up, the assembly consisting of the chamber, withdrawal tube, influent and effluent filters is sterilized by autoclaving. During this procedure a cotton plug is used in place of the rubber stopper, *St*, and the glass inlet tube for the medium is not connected to the aspirator bottle but is protected by a vial and cotton wrapping at *G*. The aspirator bottle is autoclaved separately, the end of the rubber tube, *R*, being protected by a vial and cotton wrapping.

After autoclaving, the chamber is placed in position, the clamp to the withdrawal tube closed, and a current of air passed through. An inoculating suspension of cells from an agar slant is introduced through the uppermost opening and the cotton plug replaced by a sterile rubber stopper, *St*, fitted with a glass rod for ease in handling. The rubber tube, *R*, from the aspirator bottle is attached to the glass tube, *G*, with aseptic precautions.

The whole procedure of sterilization is indeed cumbersome but it is required only at infrequent intervals of a month or more. New aspirator bottles of medium can be inserted by carefully removing the rubber tube of the old bottle, flaming the glass tube, *G*, and slipping on the sterile rubber connection of a new bottle. In operation a sample (about 1 ml.) is withdrawn daily into a flask of glucose-peptone broth as a check against bacterial or mold contamination. Many cultures have been run for a month or more without contamination.

Illumination is provided by four tubular fluorescent or tungsten filament lumiline bulbs spaced symmetrically around the chamber. (Only one lamp is shown in the diagram.) Short period variations in light intensity are minimized by use of a voltage stabilizer which delivers  $115 \pm 1.0$  volts to the lamps. Light intensity may be varied by changing the distance of the lamps from the chamber or by surrounding the lamps with sleeves made of wire screen.

Constant density of population is maintained by adding fresh medium as the culture grows. The solenoid valve, *SV*, is operated by a photometric device. Two photocells,<sup>3</sup> *P*<sub>1</sub> and *P*<sub>2</sub>, are introduced into the current-balancing circuit recommended by Wood (1934) by connecting terminals of opposite polarity. The meter,<sup>4</sup> *G*, is a spotlight galvanometer which has a No. 922 RCA phototube mounted in place of the usual glass scale. The phototube

<sup>3</sup> General Electric rectangular, barrier-type photocells  $\frac{29}{32}$  inch  $\times$   $1\frac{45}{64}$  inch.

<sup>4</sup> Rubicon No. 3402; period 3.7 seconds; *R* = 335 ohms; CDRX = 3900 ohms.

feeds into an electronic relay employing a No. 2051 vacuum tube. The arrangement is such that an increase in light on the phototube energizes the relay. The relay operates the solenoid valve, *SV*.

The two photocells look at the same light bulb.  $P_1$ , the inside photocell, is screened by the annulus of algal suspension.  $P_2$  is screened as desired by insertion of one or more uniformly exposed lantern slides, *S*. The outside photocell,  $P_2$ , is jacketed by a glass condenser through which is circulated the same constant temperature water used for the chamber; photocell and jacket are partially enclosed within a box arranged so that light enters only from the direction of the one light source. When low population densities (1 to 2 c.mm. cells per cc.) are desired, the inside photocell,  $P_1$ , is also screened with a piece of white paper so that its maximum output is about 50 microamperes. This decreases the sensitivity but greatly improves the stability of the photocell circuit.

In order to minimize ambient light effects on the photocells, the apparatus is partially enclosed in a box 30 x 30 x 36 inches high, painted white inside. The chamber just barely projects through a hole in the top of the box; and the air filters, solenoid valve, and aspirator bottle of medium are all mounted on top. The front of the box is a sheet of plywood with holes provided for ventilation and is easily removed when a sample is to be withdrawn.

In practice the original inoculum is allowed to grow until a desired population density is reached and the photocells are then balanced by inserting screens in front of the outside photocell. Thereafter a sample is withdrawn at a fixed time each day. The air flow is temporarily stopped and a small sample (~1 ml.) collected in a flask of sterile broth as a check against contamination. The rest of the culture is then withdrawn down to a mark placed a centimeter or so above the top of the inside photocell. This leaves an inoculum (50 to 100 ml. of suspension) for the next day's growth. As the algae multiply illumination on the inside photocell,  $P_1$ , is reduced. An off-balance current flows through the primary photocell circuit causing the galvanometer light spot to move across the phototube. The electronic relay then actuates the solenoid valve and allows new culture medium to flow in, diluting the algae and increasing the illumination on  $P_1$  until a zero current again obtains through the galvanometer. In this way the algal suspension "grows" up the chamber. The design of the apparatus is such that conditions of illumination are independent of the total amount of the culture.

If samples are harvested from a culture at equal intervals, then the amount of the sample is an index of the rate of growth. It is also possible to harvest samples at any other desired time, provided that the culture is not allowed to run over. Daily samples of 200 to 300 ml. containing 200 to 600 c.mm. of algal cells are easily obtained.

### *Operation of the Apparatus*

Satisfactory operation of the entire apparatus depends upon the stability and sensitivity of the primary photocell circuit. By maintaining the two photocells at constant temperature and keeping their illumination at a low level good stability may be obtained over a period of weeks. The over-all sensitivity is such that the solenoid never allows more than 1 per cent of the volume of the culture to flow in during one relay cycle.

One unit of the apparatus has been in operation for over a year and a second unit for about 6 months. Numerous difficulties have arisen. The present procedure of maintaining pure culture conditions is the result of a gradual improvement in technique. Development of an apparatus for producing a reliable gas mixture gave considerable trouble. In general the mechanical problem has been one of maintaining stability over long periods of time.

Several modifications in the preparation of culture media have been necessary. Originally we used a Knop's solution from which calcium was omitted (0.010 M  $\text{MgSO}_4$ , 0.012 M  $\text{KNO}_3$ , 0.009 M  $\text{KH}_2\text{PO}_4$ ,  $1.0 \times 10^{-5}$  M ferric ion). Micro elements were provided by the addition of 1.0 ml. per liter each of the A5 and B6 solutions of Arnon (1938). These provide in the final medium 0.5 parts per million B, 0.5 ppm. Mn, 0.05 ppm. Zn, 0.02 ppm. Cu, and 0.01 ppm. each of Mo, V, Cr, Ni, Co, W, Ti. In this medium contained in a pyrex aspirator bottle there would develop in time a fine white precipitate. Concurrently, a culture provided with the medium would show a decreased rate of growth and lowered capacity for photosynthesis. Subsequently, we have purified the major salts by the adsorption procedure of Stout and Arnon (1939), increased the iron concentration to  $13.3 \times 10^{-5}$  M, and added sodium citrate to give 0.00056 M citrate as used by Hopkins and Wann (1927). With these modifications the culture media will remain clear indefinitely and rate of growth and capacity for photosynthesis are as great as obtained by any other method of preparation of medium.

It has also been found that after insertion of a fresh bottle of medium the rate of growth and capacity for photosynthesis may be lowered for a day or so. It has been possible to demonstrate that the Knop's solution in contact with the rubber tubing leaches some toxic materials out of the rubber during autoclaving. This difficulty has been minimized by (1) using rubber tubing previously boiled in dilute alkali and leached out in distilled water and (2) running several hundred milliliters of medium from a fresh bottle out into a sterile flask before attaching the rubber tubing to the glass inlet tube of the apparatus.

Presented in Table I are typical data obtained on a culture of *Chlorella pyrenoidosa* (Emerson's strain) over a period of 3 weeks. The scanty data on the first eleven samples are omitted since rate of growth and capacity for photosynthesis were apparently limited by iron concentration. After the ninth sample a new bottle of medium containing  $9.0 \times 10^{-5}$  M iron was inserted.

From the twelfth sample on all data are presented, though in several cases data are believed invalid due to serious failure of the temperature control

TABLE I

*Typical Data Obtained on Chlorella pyrenoidosa Grown in the Continuous Culture Apparatus*

Temperature 25.05°C. Light intensity ~160 foot-candles as provided by four 20 watt "Daylight" fluorescent bulbs mounted 27 cm. from the chamber.

Sample No.	Sample size	Population		Maximum apparent rate of photosynthesis	
		Cells	Cell volume	Buffer 9	Buffer 11
	ml.	$10^8/c.mm.$	$c.mm./cc.$	$c.mm. O_2/min./c.mm. cells$	$c.mm. O_2/min./c.mm. cells$
12	216	—	1.33	—	—
13	—	0.0118	1.33	0.65	0.70
14	—	0.0120	1.33	0.62	0.66
15	—	0.0118	1.33	0.62	0.66
16	212	0.0121	1.33	0.64	0.68
17	216	—	—	—	—
(Added additional A <sub>5</sub> and B <sub>6</sub> to medium to give 1.5 ml./liter of each)					
18	224	—	1.33	0.63	0.69
(Added iron to give total concentration of 19. $\times 10^{-5} M$ )					
19*	211	—	—	—	—
20	226	0.0169	1.39	0.64	0.70
21	226	0.0168	1.33	0.66	0.71
(Inserted new bottle of culture medium)					
22*	123	—	—	—	—
23*	148	—	—	—	—
24	208	0.0141	1.33	0.64	0.68
25	198	—	—	—	—
26	213	—	—	—	—
27	200	—	1.31	0.63	0.67
28	205	0.0150	1.36	0.62	0.68
29	220	—	1.33	—	—
30	198	0.0180	1.36	—	—
31	202	—	1.39	0.62	0.68
32	210	—	1.39	—	—
33	198	0.0200	1.36	0.63	0.71
(Culture discontinued)					
Mean.....	210.5	—	1.346	0.633	0.687
Standard deviation.....	9.5	—	0.025	0.0125	0.017
Maximum variation.....	28.0	—	0.08	0.04	0.05

\* Temperature failure; data for this sample not valid.

mechanism. Mean values, maximum variation, and the standard deviation are given at the foot of each column.

Samples were removed from the chamber at 24 hour intervals and 79 ml. of suspension left for inoculum each time. The mean sample size (omitting



samples 19, 22, and 23) of 210.5 ml. indicates a multiplication rate of 3.66 per 24 hours under the conditions employed. There is a slight downward trend in the data on sample size, probably reflecting a decay in light output of the lamps with time.

Densities of population of the samples were determined by means of hemocytometer counts (giving cells per c.mm.) and by centrifuging to give packed cell volumes (c.mm. cells per cc.). Hemocytometer counts were quite variable, probably reflecting to some extent the variations in culture media used. Each count represents the mean value from a sample of about 1000 cells so that the experimental error is about 5 per cent. Of all the data, these show the greatest variation.

Packed cell volumes were determined by centrifuging 15.0 ml. of suspension in a 15 ml. centrifuge tube. The packed cells were resuspended in a little of the same medium and transferred to a Bauer and Schenk tube of 3 ml. capacity and graduated in 0.004 cc. divisions. 1.33 c.mm of cells per cc. corresponds to 5.0 divisions which can be estimated to about 0.1 division. Constant values for the packed cell volume are obtained between 15 and 25 minutes centrifuging at a relative centrifugal force of 2150. It will be seen that the packed cell volume (c.mm. cells per cc.) is quite uniform with a maximum variation of about 6 per cent and a standard deviation of about 2 per cent.

Capacity for photosynthesis of the cells was determined by the Warburg technique, using the Warburg buffers 9 and 11 to provide approximately saturating concentration of carbon dioxide, a temperature of  $25 \pm 0.05^\circ\text{C}.$ , and a saturating light intensity. The light sources used were a grid of white fluorescent tubing ( $\sim 1000$  foot-candles) or two 60 watt lumiline bulbs ( $\sim 450$  foot-candles) immersed in the water bath just below the vessels. No differences in photosynthesis rates produced by the two sources can be detected when they are used at full intensity. For the manometric measurements a 5.0 ml. aliquot of the sample was pipetted into a 15 ml. graduated centrifuge tube. The cells were centrifuged out, suspended in distilled water, centrifuged out, suspended in Warburg buffer 9 ( $0.015 \text{ M K}_2\text{CO}_3 + 0.085 \text{ M KHCO}_3$ ), centrifuged out, and suspended in buffer 9 to give 15.0 ml. 5 ml. of this suspension were pipetted into each of two rectangular Warburg vessels of about 10 ml. volume. By a similar procedure 5.0 ml. of a suspension of cells in buffer 11 ( $0.005 \text{ M K}_2\text{CO}_3 + 0.095 \text{ M KHCO}_3$ ) were delivered to each of two other vessels. Rate of photosynthesis was determined graphically by plotting 5 minute readings taken over a period of about an hour. The rate in terms of  $\Delta\text{mm. pressure per minute}$  was multiplied by the vessel constant and divided by the volume of cells used to obtain c.mm  $\text{O}_2$  per min. per c.mm. cells. Duplicate rates generally agree within a few per cent; the averages of the duplicates for each buffer mixture are the values listed in the fifth and sixth columns

resistance (in 0.1 N KCl). The membrane connected to a rubber stopper carrying a capillary manometer tubing was placed in a beaker of water and the meniscus in the manometer was observed. After 24 hours the rise of the meniscus amounted to 100 mm. For the capillary used, this corresponded to a volume transport of 100 mm<sup>3</sup> water during this period. When this experiment was performed with a dried collodion membrane of the conventional type no water movement was observed even after several days. Similar results were obtained with electrolyte solutions,<sup>15</sup> no detectable amount of electrolyte appearing in the water.

## V

The megapermselective collodion membranes fulfill almost completely the requirements originally prescribed for the desired selectively (cat) ion-permeable membranes. The membranes are extremely impermeable to anions, as measured by the characteristic concentration potential and direct exchange studies. The resistance of the membranes can be made almost as small as desired, the only disadvantage being that membranes of very low resistance (<10 ohms in 0.1 M KCl solution) in most instances are fairly weak and will stand only careful handling. Membranes of higher resistance, though weaker than unoxidized membranes, are sufficiently strong for considerable handling. The membranes are of a well defined shape and perfectly smooth; they can be stored in water for a long time (several weeks) without significant change in their ionic selectivity. Furthermore the method of preparation is simple and reproducible.

To show the improvement that has been made in the electrical properties (ionic selectivity and absolute permeability) of the membranes of the "dried" type, a comparison of several kinds of frequently used "dried" collodion membranes is presented in Table IV. The potentials and resistances were determined as described before. The membranes were all of nearly the same size; their thickness was 5 to 10  $\mu$  except for the megapermselective membranes which were 30  $\mu$  thick.

Table IV brings out clearly the advantages of the megapermselective membranes over the more conventional types of dried collodion membranes. Membranes which combine extreme ionic selectivity with any desired resistance, from 0.5 $\Omega$  to several hundred ohms per 50 cm.<sup>2</sup>, may be prepared now at will as may be required for different purposes.

<sup>15</sup> That anomalous osmosis may in these cases have been a contributing factor is not entirely ruled out. Theoretical considerations strongly favor the assumption that anomalous osmosis has not contributed to a significant extent to the observed effect. Only extensive experimental work can decide this question in a definite manner.

## VI

The availability of the megapermselective collodion membranes opens up a wide field for further investigation.

The new type membranes have already been used with considerable success in the electrometric titration of the alkali ions and of  $Mg^{++}$ .<sup>16</sup>

TABLE IV

*A Comparison of Electromotive Behavior and Resistance of Several Kinds of Conventional "Dried" and of "Megapermselective" Collodion Membranes*

Description of membrane	Characteristic concentration potential 0.1 M KCl/0.01 M KCl	Resistance in 0.1 M KCl	Remarks
	<i>mv.</i>	$\Omega/50\text{ cm.}^2$	
Mallinckrodt "Parlodion," commercial preparation	25-40	>10000	Resistance cannot be adjusted at will
Baker Collodion U.S.P., commercial preparation	40-45	>10000	
Schering-Kahlbaum "Celloidin"*	40-53	~1500	
Crude collodion†	45-50	~2000	
Oxidized Collodion, Baker collodion U.S.P., oxidized with NaOBr	50-55	>10000	
Oxidized collodion, Baker collodion U.S.P., oxidized with NaOH	50-55	>10000	
"Oxidized" membranes, dried membranes oxidized with NaOBr	51-53	~1000	Resistance can be adjusted at will
"Megapermselective" membranes	54-55.1	< 1 to > 100	

\* The concentration potential of membranes prepared from this collodion drops appreciably on prolonged contact with water or electrolyte solutions.

† Obtained through the courtesy of the Monsanto Chemical Company, St. Louis, Mo.

In the study of ionic permeabilities through negative membranes it will be possible to extend the investigation to membranes of graded selectivity and also to the bivalent ions, the behavior of which is little known at present.

The megapermselective membranes also will allow the solution under well defined conditions of one of the most urgent problems in the whole field of membrane permeability; namely, the systematic comparison of the permeabilities of ions with those of non-electrolytes. At present very little is known about this problem, since the absolute permeability of membranes of distinct ionic selectivity was heretofore much too small to allow extensive and conclusive experimentation.

<sup>16</sup> Sollner, K., *J. Am. Chem. Soc.*, 1943, **65**, 2260.

Whether or not the megapermselective membranes may be suitable for the measurement of the osmotic pressure of electrolytes and low molecular weight non-electrolytes, has not been tested. Preliminary experimental evidence indicates that they may be used for the separation by ultrafiltration of low molecular weight solutes from the solvent.

A further fruitful field of investigation can be seen in the study of membrane equilibria which involve only strong inorganic electrolytes. Membranes of this type frequently have been discussed in the physiological literature but never have been realized experimentally in a satisfactory manner.

These and related problems are at present under investigation and we hope to be able to report the results in the near future. To discuss the possible usefulness of the megapermselective membranes for practical purposes in the laboratory or their application to industrial processes is outside the scope of the present paper.

#### SUMMARY

1. The electronegative membranes described in the literature which show a high degree of ionic selectivity (permitting cations to pass and restricting the anions) have serious shortcomings: their absolute permeability is extremely low, much too small for convenient experimentation; their ionic selectivity in most cases is not as perfect as would be desirable, and is moreover adversely affected by prolonged contact with electrolyte solutions.

2. A method has been worked out to prepare membranes substantially free from these defects. Porous collodion membranes were cast on the outside of rotating tubes and then oxidized with 1 M NaOH. By allowing the oxidized porous membranes to dry in air on the tubes membranes of desirable properties are obtained. These membranes are smooth, have a well defined shape, and allow considerable handling without breaking.

3. This new type membrane when tested for ionic selectivity by the measurement of the "characteristic concentration potential," consistently gives potentials of 54 to 55 mv., the maximum thermodynamically possible value (at 25°C.) being 55.1 mv. This high degree of ionic selectivity is not lost on prolonged contact with water, and is only very slowly affected by electrolyte solutions.

4. The absolute permeability of the new type membranes can be varied over a very wide range by changing the time of oxidation. Under optimum conditions membranes can be obtained with a resistance in 0.1 N KCl solution of only 0.5 ohms per 50 cm.<sup>2</sup> membrane area. The absolute rate of cation exchange through these membranes between solutions of different uni-univalent electrolytes is very high, in one case, *e.g.* 0.9 m.eq. cations per 4 hours, the anion leak being 0.02 m.eq. Thus, the absolute permeability of the new

type membranes is two to four orders of magnitude greater than the permeability of the dried collodion membranes and the oxidized ("activated") dried collodion membranes used heretofore. Because of the characteristic properties of the new type membranes the term "*megapermselective*" (or "*permselective*") collodion membranes is proposed for them.

# THE RHEOLOGY OF THE BLOOD

## IV. THE FLUIDITY OF WHOLE BLOOD AT 37°C.\*

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The fluidity of blood serum and of fibrinogen solutions has been briefly referred to (1a). We will now consider a few data on the fluidity of whole blood of persons of different age and sex, at various times of day and under different conditions of health, with different nourishment and exercise in order to have in mind the conditions that affect the property. We will choose for our first consideration data obtained with the Hess viscometer by a few workers who accumulated a considerable mass of data each. We assume the fluidity of water at 37° to be 144 rhes.

Since the fluidity of the whole blood of healthy adult males is lower than that of youths or of females and less changeable than that of diseased persons, we will first (Table I) study the data for forty-eight healthy males who were over 14 years of age studied by Blunschy (2) in 1908. They are arranged in the order of increasing hemoglobin percentage content of the blood, the ages also being given. It is observed that the fluidity increases with the hemoglobin content and we have fitted the following formula to the data:

$$\phi = 53 - 0.24 H \quad (1)$$

where  $H$  is the hemoglobin content by the Sahli method, 16.1 gm. per 100 gm. of blood being considered normal, and 53 is the fluidity which the blood would have if the hemoglobin content were reduced to zero; i.e., plasma. On the other hand, the equation points to a hemoglobin content of 220 for a fluidity of zero, provided of course that the equation is valid for extrapolation. The average deviation between the observed and calculated values is 3.3 per cent. Since the hemoglobin content is obviously not correct to two significant figures, this is perhaps all that can be expected. Assuming the average hemoglobin content of the healthy adult male to be  $90_{\pm 7}$ , the normal fluidity of this individual should be 31.4 rhes. Dividing the cases in Table I into four groups of twelve, it may be observed that in the first group the average age is 24 years, in the second 34, in the third 31, and in the fourth 30. It appears that those of this first group are low in hemoglobin content and somewhat low in age, showing a connection between hemoglobin content and age, which is more marked as the age is lowered; nevertheless the formula for calculating the fluidity applies

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TABLE I

*The Relation between the Fluidity and the Hemoglobin Content of the Blood of Healthy Males  
above 14 Years of Age*

After Blunschy (2).

Age	$\phi$	$H$ obs.	$\phi$ calc.	Deviation
15	37.2	70	36.2	-1.0
16	35.8	72	35.7	-0.1
32	33.6	75	35.0	+1.4
16	35.1	80	33.8	-1.3
32	34.5	80	33.8	-0.7
20	34.0	80	33.8	-0.2
16	33.7	80	33.8	+0.1
16	33.3	80	33.8	+0.5
43	33.1	80	33.8	+0.7
21	31.9	80	33.8	+1.9
20.5	32.7	80	33.8	+1.1
38	32.7	82	33.3	+0.6
58	31.8	82	33.3	+1.5
23	32.9	85	32.6	-0.3
22	32.5	85	32.6	+0.1
20	32.4	85	32.6	+0.2
63	32.2	85	32.6	+0.4
20	32.2	85	32.6	+0.4
27	32.1	85	32.6	+0.5
33	32.1	85	32.6	+0.5
33	31.9	85	32.6	+0.7
23	31.7	90	31.4	-0.3
25	31.6	90	31.4	-0.2
65	31.2	90	31.4	+0.2
19	31.1	90	31.4	+0.3
18	31.0	90	31.4	+0.4
21	31.0	90	31.4	+0.4
21	30.7	90	31.4	+0.7
28	30.7	90	31.4	+0.7
33.5	30.6	90	31.4	+0.8
56	30.2	90	31.4	+1.2
32	30.0	90	31.4	+1.4
60	30.0	90	31.4	+1.4
40	31.0	93	31.7	-0.3
29	31.6	95	30.2	-1.4
22	31.3	95	30.2	-1.1
31	30.8	95	30.2	-0.6
21	30.7	95	30.2	-0.5
30	30.0	95	30.2	+0.2
21	29.5	100	29.0	-0.5
27	29.1	100	29.0	-0.1
29	28.8	100	29.0	+0.2
27.5	27.4	100	29.0	+1.6
43	29.7	105	27.8	-1.9
24	27.9	110	26.6	-1.3
30	25.6	110	26.6	+1.0
37	25.5	110	26.6	+1.1
42	24.1	110	26.6	+2.5

well, showing that the variation in the hemoglobin percentage may account for almost the entire changes in fluidity encountered in healthy adult males.

### Youths

Blunschy (2) studied seven cases of healthy males below 14 years of age. The fluidity in all cases is much higher, 1.2 rhes, than for adults but the hemoglobin content is also uniformly lower, although not quite enough lower to account for all of the elevation of the fluidity. In all except one case, as shown in Table II, the calculated fluidity is lower than the observed, the average deviation being 1.0 rhes but without any distinct trend.

Schukowa-Florensova (3) reported the average viscosities of normal healthy children of different ages. The percentages of hemoglobin were not reported,

TABLE II

*The Relation between the Fluidity and the Hemoglobin Content of the Blood of Healthy Males below 14 Years of Age*

Age	$\phi$	$H$ obs.	$\frac{\phi \text{ calc.}}{\text{Equation (1)}}$	Deviation $\pm 1.2$
8	37.6	68	36.7	+0.3
6	39.9	70	36.2	-2.5
4	37.6	70	36.2	-0.2
6	36.2	70	36.2	+1.2
12	35.9	70	36.2	+1.5
6	36.7	72	35.7	+0.2
5	36.9	75	35.0	-0.7
Average .....				1.0

so that the above formula cannot be applied to them. The fluidities, however, are given in Table III since they do prove that the fluidity of the blood of youths falls nearly linearly from the earliest years, according to the formula

$$\phi = 47.3 - 0.48 y \quad (2)$$

where  $y$  is the age in years. If the high fluidity in these early years were entirely to be ascribed to low percentage of hemoglobin in the blood, the hemoglobin could be calculated also by the formula (1), as given in the fifth column of Table III and it would show a nearly steady rise from 1 year on.

It is important to get these data for hemoglobin. According to Mayers (4), the amount of hemoglobin in the blood of youths increases from the age of 1 year to 16 when it becomes constant and remains so until about 75 years of age. But according to him, up to 1 year the hemoglobin decreases steadily and rapidly from birth.



*Women and Girls*

The data of Blunschy for the fluidity of the whole blood of healthy women and girls given in Table IV are confined to sixteen cases. The observed fluidity is on the average of 0.7<sub>6</sub> rhes higher than the value calculated by equation (1) for adult men. This small difference may be experimental error but we will regard it. The average woman has a blood with a hemoglobin content 78<sub>±3</sub> which would correspond to a fluidity for males of 34.3 rhes. If we increase this by 0.7<sub>6</sub>, we arrive at the expected average for women of 35.1 rhes, to be compared with the expected average for men of 31.4 rhes. This large difference

TABLE III

*The Fluidity of the Whole Blood of Healthy Children of Various Ages*

After Schukowa-Florensowa (3).

Age	$\phi$ obs.	$\phi$ calc. Equation (2)	Deviation	$H$ calc. Equation (1)
0.6	48.0	47.0	-1.0	—
1	45.6	46.8	+1.2	29.1
2	45.9	46.3	+0.4	27.8
4	45.3	45.4	+0.1	30.3
5	45.5	44.9	-0.6	27.8
6	44.9	44.4	-0.5	32.0
7	43.3	43.9	+0.6	38.7
8	43.5	43.5	0.0	37.9
9	43.2	43.0	-0.2	39.2
10	43.9	42.5	-0.4	40.4
11	42.2	42.0	-0.2	44.2
12	39.8	41.5	+1.7	53.4
13.5	41.8	40.8	-1.0	45.0
15.5	39.5	40.0	+0.5	54.7

is due to the much lower hemoglobin content of 78 in the blood of the women tested. This difference between the two sexes accords with general experience. In the last column of Table IV, there is given the deviation as a correction for sex.

*The Serum and Plasma*

It is clear that the hemoglobin content of the blood of healthy persons varies widely, irrespective of age or sex and it has such an important effect that no study of the rheology of the blood can neglect it; however, that does not mean that the shape and number of the erythrocytes is not important or that the leucocytes are without rheological effect. The question naturally suggests itself as to whether the effect of the variation in blood cells should not be eliminated for comparative purposes by reducing the blood to a *standard condition*

of either a normal number of blood cells or perhaps still better to a plasma without blood cells. Important as are the blood cells, the fluidities of the serum and plasma are important and they can be determined with greater ease and precision than can that of the whole blood. The measurement of the fluidity of the whole blood would perhaps best be used to determine the hemoglobin or, more exactly, the rheological constant of the red cells. It is often stated that the viscosity of the serum and plasma vary relatively little. This statement is worthy of examination because as a result of the digestive processes,

TABLE IV

*The Relation between the Fluidity and the Hemoglobin Content of the Blood of Healthy Women and Girls*

After Blunschy (2).

Age	$\phi$ obs.	$H$ obs.	$\phi$ calc. Equation (1)	Deviation
49	43.6	55	39.8	-3.8
6.5	39.8	60	38.6	-1.2
5.5	38.7	68	36.7	-2.0
20.5	37.9	70	36.2	-1.7
21	36.9	70	36.2	-0.7
17	35.1	75	35.0	-0.1
32	34.7	80	33.8	-0.9
32	34.3	80	33.8	-0.5
44	31.4	80	33.8	+2.4
20	34.0	85	32.6	-1.4
55	32.7	85	32.6	-0.1
21	33.2	85	32.6	-0.6
40	33.0	85	32.6	-0.4
40	32.4	85	32.6	+0.2
31	32.7	90	31.4	-1.3
65	31.2	90	31.4	+0.2
Average .....				-1.1

water and food are being taken into the blood stream at varying rates. Thus salts, both inorganic and organic, glucose, fats, and urea are present together with considerable amounts of proteins. These last are by far the most important in lowering the fluidity, the most important being the albumin and globulin (8.2 per cent), although the smaller percentage of fibrinogen (0.8 per cent) is relatively even more important. Table V after Bolle (5) shows that the plasma containing the fibrinogen, invariably has a lower fluidity than the serum. The percentage of dry solids in the serum and in the plasma is given in the third and fourth columns. These are to be corrected for salts, etc. In the ten individuals who were tested, the serum had an average fluidity of 86

rhes and the plasma a fluidity of 70 rhes. The difference of 16 rhes is due to the fibrinogen, which, however, shows a wide variation of from 10 to 20 rhes which is nearly 30 per cent.

The ratio of albumin to globulin in the serum is regarded as of importance, and it shows wide variation in different individuals; the average ratio is about 62 to 38 which is roughly 5 to 3. It is thus possible to explain the wide variations in the fluidity of serum and plasma in Table V.

Starting with water which has a fluidity of 144 rhes at 37° the salts lower the fluidity by less than 1.5 rhes, the albumin and globulin together may lower it by 58 rhes, and the fibrinogen by an additional 16 rhes. Since the fluidity of the whole blood of the healthy adult male is about 31.5 rhes, it is evident that the

TABLE V

*The Fluidities of the Serum and Plasma of Various Individuals, the Percentage of Dry Residue, and the Kjeldahl Nitrogen*

After Bolle (5).

Fluidity		Dry residue		Kjeldahl nitrogen	
Serum	Plasma	Serum	Plasma	Serum	Plasma
96	80	9.92	10.95	1.092	1.162
85	72	9.704	10.33	1.083	1.218
90	80	8.27	8.47	1.22	1.24
85	72	10.51	—	1.171	1.265
88	72	10.04	11.22	1.157	1.288
85	72	9.87	10.92	1.30	1.33
82	65	10.89	13.46	1.273	1.358
90	69	10.01	11.48	1.269	1.372
80	60	11.58	13.42	1.279	1.395
85	63	11.92	13.02	1.220	1.407

actual lowering of the fluidity by the salts and proteins (73.5) is very much more than the lowering due to the blood cells (39 rhes). This is a very impressive fact because the blood cells make up 30 per cent of the volume of the blood. The erythrocytes must therefore possess a rather high mobility in their interior and rheologically they must be regarded as almost totally different from solid particles in suspension, perhaps more like an emulsion with the internal phase having high fluidity. This is the more remarkable since the red corpuscle is 32 per cent by weight hemoglobin which is said to be not in true solution. The flexibility of the red corpuscle is readily observed, but we are not aware that it has been measured. Also data seem to be lacking on the consistency of the contents of the corpuscle. It is well known that the organism is capable of adjusting the ratio of albumin to globulin within the body and thereby rather quickly affecting the fluidity of the blood while maintaining the water content

constant. Reiss (6) showed that the proteins can be estimated by means of the refractometer. Schorer (7) showed that globulin has a higher refractive index than albumin, and suggested a method for estimating their relative amounts. Heyder (8), at the suggestion of Nägeli (9), experimented with concentrations of these two proteins, approximating those found in the blood

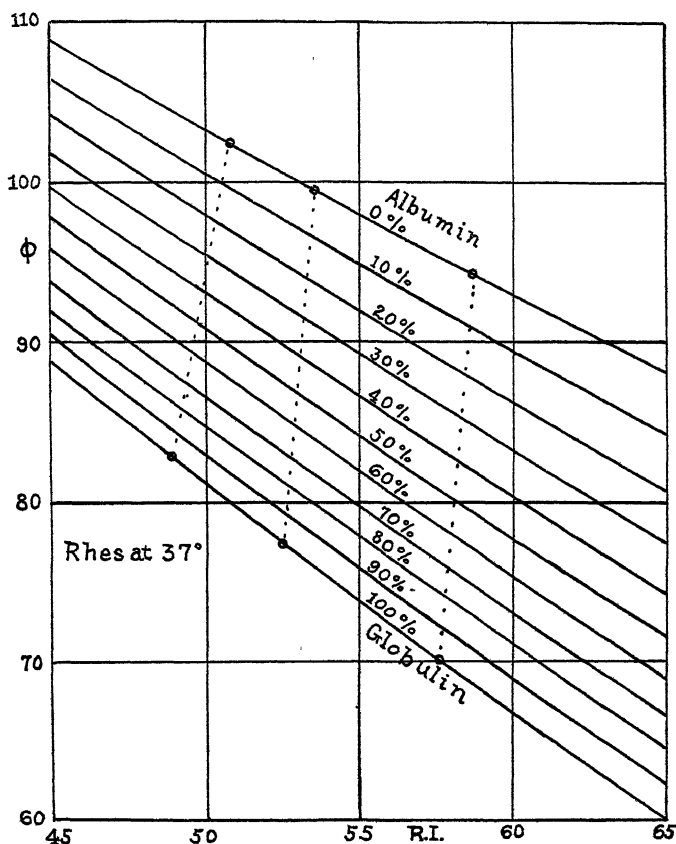


FIG. 1

serum; *viz.*, 6, 7, and 8 per cent. Nägeli constructed a family of curves, R. I. *vs.* percentage of globulin and also relative viscosity *vs.* percentage of globulin. The former were nearly linear while the latter were strongly curved. It is of interest to note that on plotting the fluidity *vs.* percentage of globulin, the curves are nearly linear, as are also the family of curves of fluidity *vs.* R. I. as shown in Fig. 1. From these data of Heyder we obtain as the concentration for zero fluidity 15.1 per cent in the case of globulin and 20.4 per cent in the case of albumin.

TABLE VI

*The Relation Between the Fluidity and the Hemoglobin Content of Diseased Persons, of Various Ages and Both Sexes*

After Blunschy (2).

Age	$\Phi$ obs.	H	$\Phi$ calc.	Deviation	Disease
59	52.4	30	45.8	-6	Ventriculitis
50	49.6	30	45.8	-4	Pernicious anemia
54	39.8	30	45.8	+7	Pleur. mitral insufficiency
53	58.8	37	43.9	-15	Ventriculitis
42	36.9	53	40.3	+3	Pulmonary tuberculosis
20.5	39.4	60	38.6	-1	Chlorose
15.5	33.2	60	38.6	+6	Mitral insufficiency with stenosis
28	43.3	65	37.4	-6	Pregnancy
22	39.5	65	37.4	-2	Chlorose
26	38.4	70	36.2	-2	Pregnancy
48	35.6	70	36.2	0	Bronchitis
66	33.9	70	36.2	+2	Mitral insufficiency
68	31.2	70	36.2	+5	Bronchitis
37	29.4	70	36.2	+7	Mitral stenosis
54	28.2	70	36.2	+8	Bronchitis
63	28.0	70	36.2	+8	Bronchitis
63	25.9	70	36.2	+10	Pneumonia croupous
50	35.6	75	35.0	-1	Bronchitis
19	32.5	75	35.0	+2	Cyanosis, congenital
28	32.3	75	35.0	+3	Mitral stenosis
65	31.5	75	35.0	+3	Mitral insufficiency
58	30.0	75	35.0	+5	Laryngitis
66	29.6	75	35.0	+5	Myocarditis
42	29.0	75	35.0	+6	Myocarditis
20	26.7	75	35.0	+8	Bronchitis acute
82	34.7	77	34.7	0	Arteriosclerosis
39	34.1	80	34.2	0	Nephritis
59	31.8	80	34.2	+2	Bronchitis
44	31.3	80	34.2	+3	Basedow's disease
68	31.0	80	34.2	+3	Myocarditis
49	30.8	80	34.2	+3	Mitral stenosis
20	28.5	80	34.2	+5	Pulmonary tuberculosis
30	28.4	80	34.2	+6	Basedow's disease
49	28.0	80	34.2	+6	Bronchitis
63	27.5	80	34.2	+6	Mitral stenosis
65	24.4	80	34.2	+10	Bronchitis
58	32.0	82	33.3	+1	Arteriosclerosis
47	31.0	82	33.3	+2	Diabetes, 3 per cent glucose
66	36.3	83	33.1	-3	Nephritis, 1 per cent albumin
26	30.5	85	32.6	+2	Syphilis II
64	28.2	85	32.6	+4	Paralysis agitans myocardial insufficiency
58	26.0	85	32.6	+7	Bronchitis
63	25.1	85	32.6	+8	Bronchitis
9	34.3	90	31.4	-3	Pulmonary stenosis
34	28.5	90	31.4	+3	Sepsis
62	26.5	90	31.4	+5	Bronchitis
42	25.3	90	31.4	+5	Syphilis II
45.5	24.6	90	31.4	+7	Bronchitis
7.5	17.3	115	25.2	+8	Pulmonary stenosis
8	11.6	140	19.4	+8	Pulmonary stenosis

*Clinical Observations*

The relation between fluidity and hemoglobin content in equation (1), appears to make it possible to compare samples of the whole blood of healthy persons above 14 years and expect concordance of about 3 per cent. Blunschy

TABLE VII

*The Relation between the Fluidity and the Hemoglobin Content of Diseased Persons, of Various Ages and Both Sexes but Neither Stated*

After Bolle.

$\Phi$ obs.	$H$	$\Phi$ calc.	Deviation	Disease
55	7	51	-4	Cancer of uterus
72	15	49	-33	Pernicious anemia
60	20	48	-12	Severe anemia
63	20	48	-15	Severe anemia
63	28	46	-15	Anemia after confinement
45	37	44	-1	Tuberculosis of lungs
40	40	43	+3	Pernicious anemia
50	40	43	-7	Anemia after confinement
42	45	42	0	Typhus
41	45	42	+1	Angina
34	50	41	+7	Arthritis gonorrhea
37	52	40	+3	Neurasthenia
37	55	40	+3	Diabetes
40	55	40	0	Pleurisy
42	55	40	-2	Sepsis after confinement
41	55	40	-1	Myocarditis
44	58	39	-5	Chlorosis
36	60	39	+3	Tuberculosis of lungs
37	60	39	+2	Tuberculosis of lungs
40	62	38	-2	Tuberculosis of lungs
31	70	36	+5	Neurasthenia
34	70	36	+2	Neurasthenia
37	70	36	-1	Tuberculosis of lungs
34	72	36	+2	Healthy man
37	75	35	-2	Ventricular ulcer
39	75	35	-4	Rheumatism of joints
41	75	35	-6	Parametritis
40	75	35	-5	Syphilis III
34	80	34	0	Healthy man
33	80	34	+1	Healthy man

has given data for fifty diseased persons and Bolle for thirty more, giving the ailment as well as the fluidity and hemoglobin content. Three persons on Bolle's list are put down as healthy and serve as norms to compare with the previous list. Blunschy's data (Table VI) show observed fluidities which are

13 per cent below the average found for healthy persons, but this figure is of little or no importance because the fluidity was found in many cases to be higher than the expected value. It is concluded that disease may affect the validity of the formula, but the effect is dependent upon the nature of the disease. Blunschy cites cases of polycythemia with a low fluidity. No. 1, a boy of 8, red cell count 9,600,000 and fluidity 11.6 rhes; No. 2, a boy of 8.5, with red cell count of 6,970,000, fluidity 17.2 rhes; and No. 3, a boy of 9, with a red cell count of 4,680,000, fluidity 35.5. All three walked up two flights of ten steps and down again from six to ten times after which the fluidity of the blood was found to be 10.6, 17.0, and 25.9 rhes respectively. There was in each case a loss in fluidity presumably due to the effect of carbon dioxide in swelling the erythrocytes. But the inhalation of 25 liters of oxygen caused the fluidity to rise to 12.4 rhes with No. 1 and to 18.3 rhes with No. 2. It is noted that the loss in fluidity by No. 3 in his sudden and severe exertion was 22 per cent. Bolle's data in Table VII give cases of cancer and anemia in which the fluidity of the blood is considerably greater than the calculated value. The average percentage deviation between the observed and calculated values has risen from 3.3 to 13.1 per cent, but even with diseased persons there is evidence of the predominating effect of the hemoglobin content, the data being arranged in the order of their increasing observed value. The increased deviation is proof that other factors than the hemoglobin content must be considered. The three boys referred to, indicate that the condition of aeration of the blood is important. It is therefore not surprising to find the fluidity lower than the expected values in bronchitis, pneumonia, and tuberculosis. It is noted that in a case with 3 per cent of glucose (Table VI) the effect on the fluidity is scarcely noticeable.

#### *Time of Day, Age, and Sex*

It is not without interest to compare now the fluidities and hemoglobin values of healthy persons of both sexes and all ages, during the hours of the day, using the data of Blunschy, Table VIII.

From the very limited data available certain provisional conclusions may be reached. From birth to 10 years the hemoglobin is low and the fluidity high. From 14 years on, the fluidity of the blood of men is on the average appreciably constant, whereas the fluidity for women is higher until after the menopause, when it is approximately the same as for men. This change is due to a rise in hemoglobin occurring after the menopause. Particularly on this point additional data are needed.

Six persons were tested by Blunschy over the waking hours of a day, two men and four women. The first test was at 7:30 followed by breakfast (B) at 8:00; they were tested again at 11:00 before lunch (L) at 12:00 M. They were again tested at 1:30 and 3:00. They were served coffee (C) apparently at 3:30 and another test was made at 4:00. A test was made at 6:00 before supper (S) at

6:30 and the final test was made at 9:00. The results for the different individuals vary but they are in general agreement, the averages only being given in Table IX.

TABLE VIII  
*The Relation of Fluidity to Time of Day, Age, and Sex*  
After Blunschy (2).

	Age in years							
	0-9.9	10-13.9	14-19.9	20-29.9	30-39.9	40-49.9	50-59.9	60-
Women	Fluidity							
	39.2	—	35.1	35.5	33.9	35.1	32.7	31.2
	Hemoglobin							
	64	—	75	77.5	83	76	85	90
17	No. of cases							
	0	0	1	4	3	4	1	1
	Fluidity							
	37.5	34.0	33.7	31.4	30.7	29.5	31.0	31.2
Men	Hemoglobin							
	65	78	77	90	91	97	86	88
	No. of cases							
	6	2	6	21	11	4	2	3

TABLE IX  
*The Change of Fluidity of the Blood with the Hours of the Day*  
After Blunschy (2).

Hours.....	A. M.				P. M.							
	7:30	B	9:00	11:00	L	1:30	3:00	C	4:00	6:00	S	9:00
Fluidity, rhes.....	29.6		31.9	31.5		33.0	32.2		32.4	32.5		32.2

It was observed by Blunschy that on rising, "*Aufstehen*," the fluidity was invariably lower by about 10 per cent, which does not take place when persons remain in bed. This has not been explained, but with the increased activity of the heart on rising one is inclined to inquire as to the possible chemical reduction of the blood causing the swelling of the red blood cells.



During the night the blood shows a marked loss in fluidity due perhaps partly to dehydration and perhaps partly to lowered respiration and aeration when reclining. At any rate the marked rise in fluidity which uniformly occurs after arising, averaging 10 per cent, is worthy of note. Arising in the morning changes the bodily tempo, it causes changes in hydrostatic pressure which may cause a certain amount of seepage of liquid into the tissues, and thus affect the concentration of the blood, but this seems of less importance. With breakfast the fluidity rises and then falls, with lunch it again rises and falls. It is not evident whether the fluidity rises and falls after supper or not.

### *Dehydration of the Blood*

The fluidity of the blood follows a somewhat zigzag course during the day, the fluidity being increased after meals according to Burton-Opitz (10), Bence (11), and Breitrner (12). We will again cite an experiment of Blunschy. After breakfast at 10:00 a.m. five persons were tested and then again at 11:30 a.m. There was during this period a loss of fluidity of 2.2 per cent. They were then served lunch but without soup or beverage and at 12:45 again tested, when the fluidity of the blood of each had increased, the average increase being 7.5 per cent. It may be argued that since food contains a very high percentage of water or the elements of water these facts may be explained by the hydration of the blood. It is not so easy to explain why warm water increases the fluidity more than cold water and beer is without effect. The fluidity of the blood is due to the water present, but the body is able to excrete any excess of water, therefore we may well hesitate to reach conclusions from scanty data until the condition of hydration of the blood is known. The blood is almost certainly dehydrated in the morning on rising, hence it is significant that there should invariably be a rise in fluidity after breakfast. It is of little importance that it turns out that later in the day when the blood is normally hydrated, the drinking of beer is without effect, on the fluidity. Blunschy found that in 12 cases the diuretic, caffeine, caused a lowering of the fluidity of the blood, without exception.

Blunschy found the effects of work also somewhat contradictory. Five persons engaged in the heavy work of cleaning a floor with steel wool from 2:00 to 3:15 p.m., perspiring profusely, and becoming very tired. In each case the blood showed a loss in fluidity, with an average loss of 4.6 per cent. Five young men, on the other hand, were out all day on a ski trip ascending to 1500 meters, and the final test was made on returning when they had not eaten for 5 hours. The fluidity of the blood of each showed an increase, the average being nearly 13 per cent. They perspired profusely but no report is made of the amount of liquid taken in, but the elevation and the cold mountain air are factors which should be considered.

Poiseuille (13) noted that alcohol added to serum increases its viscosity and it

does the same when injected into the blood stream of animals. Nevertheless, cognac administered to fourteen young men was found by Blunschy to raise the fluidity in every case save one, with an average of 5 per cent. In this case also there is then an apparent contradiction.

Relative to the importance of the water reserve of the body, Cannon (14) quotes Rubner to the effect that through fasting, one may lose practically all of his stored glycogen, without any noteworthy consequences, all of his reserve of fat, and one-half of the protein which is either stored or built into the body structure and yet not be confronted with great danger. On the other hand, the loss of only 10 per cent of the body water is serious and a loss of from 20 to 22 per cent means certain death. In other words, while the body contains some 45 kilos of water on the average, the loss of only 5 kilos of water is fraught with danger although that is scarcely as much as the weight of the blood itself (5.5) and a loss of water which is twice that of the weight of the blood cannot be tolerated. This very impressive fact seems to have stimulated little research commensurate with its importance.

Dehydration of the body occurs under a variety of conditions, some pathological as in nausea, diarrhea, cholera, etc., others not pathological as in the case of severe burns or famishing on sea or land. Czerny (15) in 1894 observed the thickening of the blood of cats kept in a warm room without water. He noted the increase in corpuscles and suggested that death was due to the increase in viscosity of the blood. The paste-like consistency of the blood of the ill-fated passengers of the dirigible Hindenburg was noted.

### *Baths*

Considerable attention has been devoted to the supposed or real effects of sweating in various types of baths. Müller (16) claimed that air-baths caused an increase in the viscosity of the blood with an increase in blood pressure (18). Both Kundig (17) and Lommel (18) failed to confirm these claims. But decisive experiments would require further information such as the state of hydration at the beginning, the loss of weight during the experiment, etc.

### *Stasis*

The stoppage of the flow of blood, as by tying, invariably produces a considerable decrease in the fluidity of the blood. There is an increase in the hemoglobin content presumably due to the diffusion of the serum through the walls of the capillaries, which may be thought of as "leaky" tubes. But calculation of the fluidity expected for the given hemoglobin content shows that the fluidity is very much lower than can be explained by it alone. The stasis results in a drop in the pH of the blood due to the accumulation of carbon dioxide. The carbon dioxide causes a swelling of the erythrocytes and a lowering of the fluidity. On the other hand, aeration of the blood causes changes to take place

which are the reverse of these, with a decrease in the fluidity of the blood, so even in taking samples of blood for test care must be taken to avoid stasis and also a separation of blood cells from plasma. Two observations of Blunschy on stasis are given in Table X.

It is in harmony with the above, that venous blood has a higher volume of blood corpuscles than the arterial but a lower fluidity.

### *Bleeding*

The effects of loss of blood in surgical operations, venesection, or from wounds have been studied by Müller (19), Oelecker (20), and others. In artificially restoring the volume to the blood, it is necessary to maintain a certain viscosity, by adding proteins as well as physiological saline solution, and most effectively by the use of blood plasma. This subject is one needing much study. The cause of the fall in fluidity with subsequent rise on venesection seems not to be fully explained.

TABLE X  
*The Effect of Stasis in a Blood Vessel*

Subject	Before stasis				After stasis			
	$\eta$ obs.	$H$ obs.	$\Phi$ obs.	$\Phi$ calc. equation (1)	$\eta$ obs.	$H$ obs.	$\Phi$ obs.	$\Phi$ calc. equation (1)
M. B.....	4.70	80	30.6	33.8	8.9	112	16.2	26.1
A. B.....	3.94	70	36.5	36.2	6.4	90	22.8	31.4

From experiments with over two hundred patients Müller concluded that the normal operation produced first a fall in fluidity in the post-operative phase, then a slower rise during the exhaustion phase in which the fluidity would rise above normal, followed by a gradual return to normal in the recovery phase. All of these changes he found to go parallel with the number of erythrocytes present in the blood as indicated elsewhere in this paper. He also proved that the fluidity of the serum was not altered. When, however, the fluidity of the blood of the patient had been greatly altered by disease, the above sequence of events might be altered thereby.

### *Air Pressure*

Both men and other animals when subjected to changes in external air pressure for considerable periods, require acclimatization involving profound changes in the blood. According to Krogh (21), when one goes to a high altitude there is a quite immediate concentration of hemoglobin, (*cf.* Oti (22)) brought about by removal of plasma but there is subsequently a slow increase in the absolute amount of hemoglobin. If the change of environment is made

early enough in life, this increase may be adequate and without injury to normal life processes, but if made late in life, it may be necessary to change periodically to the former environment, thus proving that the acclimatization is far from complete. This subject will become of greater interest perhaps as aviation in the stratosphere becomes more common.

If it is true that men doing heavy work and athletes and soldiers called upon for sudden and extreme exertion demand more meat in their diet than do women and others doing light work, we find an explanation in the higher hemoglobin content of the blood in the former, since hemoglobin is produced in the body by eating meat, particularly liver. This raises the question as to whether persons with a high hemoglobin content of the blood can be better acclimated to high altitudes.

It appears axiomatic that the blood is as fluid as possible without loss of efficiency, because unnecessary viscosity means added work for the heart. This might indicate that women and children and even men doing light work possess a certain advantage if their normal hemoglobin content is low. But it has to be remembered that the circulatory system is more than for transportation solely; the blood is also a reservoir, since the plasma diffuses quite freely into the *milieu interne*. Thus the body is nourished and the lymph gives suppleness and softness to the tissues and skin. The proteins play an important rôle in the flow by osmosis through the intestinal walls, in the kidneys and elsewhere, maintaining the homeostasis of water in the blood. Thus the acclimatization of the animal to a new environment is not a simple matter.

### *Comparison of the Blood of Other Animals*

There have been many measurements of the viscosity of the blood of the lower animals, but recently Rhie (23) has reviewed the earlier data and made a systematic study of six species of animals, using ten individuals (five male and five female) of each species, measuring the viscosity of the whole blood, the plasma and serum as well, and the hemoglobin content. We will not attempt a detailed discussion, but such ample data make some inferences desirable.

In every species, different individuals exhibit variations of fluidity and hemoglobin content which are wide as compared with man. The average values given in Table XI, show that the blood of rabbits (I) is distinctly more fluid and that of pigs (VI) less fluid than that of the other species and in the following general order: rabbits (I), goats (II), horses (III), sheep (IV), cattle (V), and pigs (VI). Genetically the rabbit is a rodent and the others ungulates, but all are mammals and all herbivorous except the pig which is omnivorous like man. It is therefore of interest that on the basis of both fluidity and hemoglobin content men are situated between pigs and cattle. We are sorry not to include data on carnivores, birds, reptiles, and amphibians.

In each species with the exception of goats, the mean hemoglobin content of

the blood was higher in the case of the males than of the females as in man; and in every species with the sole exception of rabbits a high mean hemoglobin content corresponded to a low mean fluidity, as we have found to be the case with individual men and women. To bring this out very clearly, all of the observations were first arranged in the order of increasing fluidity. The data were divided into decades and the number of individuals in each species noted for

TABLE XI

*The Mean Fluidities at 37° of the Whole Blood of Different Species and Sexes of Mammals with Values Calculated from the Hemoglobin Content of Rhesus Using Formula  $\Phi = \Phi_1 - bH$*

Animal	Sex	H obs.	$\Phi$ obs.	$\Phi$ calc.	$\Phi_1$	b	Deviation per cent
Rabbits I	♂	11.98	42.0	34.6			
	♀	11.72	41.3	41.6			
	Average.....	11.86	41.6	34.8	52.6	0.92	4.4
Goats II	♂	10.73	37.8	37.1			
	♀	11.01	35.4	36.5			
	Average.....	10.87	36.6	36.8	57.6	1.93	5.5
Horses III	♂	11.96	34.0	34.6			
	♀	11.01	36.7	36.5			
	Average.....	11.48	35.4	35.6	37.6	0.19	10.8
Sheep IV	♂	12.37	33.2	33.8			
	♀	11.25	35.3	36.0			
	Average.....	11.81	34.2	34.9	73.8	3.35	9.5
Cattle V	♂	12.55	31.1	33.4			
	♀	10.94	31.7	37.7			
	Average.....	11.74	31.4	35.0	43.2	1.00	4.4
Pigs VI	♂	16.21	23.6	36.1			
	♀	15.71	25.1	37.1			
	Average.....	15.96	24.4	26.6	63.4	2.44	5.4

every decade as given in Table XII and the mean position for each species was calculated, which is given in the final column. It is noted that the species seem about equally separated from each other, but the separation of rabbits from the others is marked. Then the same operation was repeated except that the data were arranged on the basis of increasing hemoglobin content (Table XIII); it is seen that the mean values increase in the reverse direction now with the exception of rabbits (I), if we neglect the practical equality of (III) and (IV). The anomaly afforded by rabbits needs explanation.

There may be no apparent reason why equation (1) should apply to two in-

TABLE XII\*

*The Grouping of Animals When Arranged by Decades according to the Fluidity of the Whole Blood*

Data of Rhiel (23).

	0	10	20	30	40	50	60	Mean
I	8	2	—	—	—	—		0.7
II	2	2	3	3	—	—		2.2
III	—	4	3	—	3	—		2.7
IV	—	2	1	4	2	1		3.4
V	—	—	2	3	3	2		4.0
VI	—	—	1	—	2	7		5.0

\*The heavy bars in Tables XII and XIII are placed according to the printer's *ems* given in decades at the head of the tables thus representing the mean values for each type. One may get a graphical idea of the facts by imagining a curve drawn through the middle of the bars in succession.

TABLE XIII

*The Grouping of Animals Arranged according to the Hemoglobin Content*

Data of Rhiel (23).

	0	10	20	30	40	50	60	Mean
I	—	4	1	3	2	—		2.8
II	—	1	1	—	4	4		4.4
III	—	—	4	2	2	2		3.7
IV	—	1	1	4	2	2		3.8
V	1	3	3	1	—	2		2.7
VI	9	1	—	—	—	—		0.6

dividuals of the same species and therefore much less reason for individuals of different species unless corpuscles of similar shape and size do lower the fluidity of plasma in the same manner. This is a large subject and the data are still inadequate. We have, however, worked out constants for the equation

$$\Phi = \Phi_1 - bH \quad (3)$$

as given in the Table XI. The average percentage deviation between the observed and calculated values using these equations is 6.7 which is just twice that obtained with healthy men and equation (1). The average value of  $\Phi_1$  is 54.7 which cannot be distinguished from the 53 found for human blood. Applying the same type equation to the entire data, the percentage of error rose to 11 per cent. The equation used was  $\Phi = 58.5 - 0.20 H$ , the values of  $H$  being not the Sahli values used by Blunschy and Bolle.

#### SUMMARY

In the preceding paper (1b) a formula was developed for the lowering of the fluidity of a medium by a mixture of proteins, given the volume concentration of each and its fluidity-lowering constant. Whole blood is now shown to follow an essentially similar formula, except that the hemoglobin content is taken from the literature as the best available measure of the volume of the blood cells  $\Delta \Phi = 0.24H$ , assuming the fluidity of the medium to be 53 rhes. Age, sex, diet, barometric pressure affect the hemoglobin content of the blood, but the formula may apply to any healthy human blood to about 3 per cent. The shape, number, and size of the blood cells, if known, might help to explain discrepancies as well as the state of oxidation of the blood. In disease the discrepancy becomes much greater, suggesting the possible use of rheology in diagnosis.

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## X-RAY DIFFRACTION STUDIES ON FROG MUSCLES\*

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X-ray diffraction methods have many of the advantages of physicochemical methods. Practically speaking, they do not alter the material under investigation but still they furnish information on the chemical structure. Such methods seem, therefore, particularly suitable for studying biological material. Because x-ray diffraction methods are limited to relatively small particle size, the microscopic structure of muscle cannot be studied by this method.

X-ray diffraction studies of muscle were reported as early as 1921 (Herzog and Jancke). Techniques adequate for the study of living muscle and its reactions were developed by Boehm (1931 *a* and *b*). Boehm and Weber (1932) have successfully identified the diffraction pattern of dried muscle with that of stretched myosin threads, thus showing that most of the findings of x-ray diffraction studies in muscle refer to changes in the muscle protein. This has been confirmed by Astbury (1936). Recently reported small-angle interferences in myosin as described by Kratky, Sekora, and Weber (1943) have not yet been found in muscle fibres.

The experiments to be reported here were made with the intention of correlating x-ray diffraction findings in living and dead muscles with chemical changes in proteins. Biedermann (1927) has explained the changes in birefringence of muscle by changes in myosin. Katz (1934) has stressed the probability that anisotropy of a substance as shown by its birefringence has a parallel in its x-ray diffraction pattern. Therefore, in most cases, changes in the latter were followed up by studies with the polarization microscope. Conversely, studies were made of the effect on the x-ray diffraction pattern of substances which were known, from the work of former authors, to affect the birefringence of the muscle.

The apparatus used in these experiments was the same as that reported before, but it has been equipped with a new camera, especially constructed for this purpose. The distance between the object and the focal spot has been shortened to 60 mm.; by using extensions of various sizes, the specimen-film distance could be varied be-

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\* Parts of this paper have been brought before the Federation of American Societies for Experimental Biology (Ashkenaz, Henny, and Spiegel-Adolf, 1943; Henny, Ashkenaz, and Spiegel-Adolf, 1944).

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tween 20 and 80 mm. Thus the exposure time for a living muscle could be cut down to 3 minutes. Controls, using nickel filters and a correspondingly longer exposure, did not show appreciable difference in the diffraction pattern. Lucite stages were used to support the muscle in a horizontal position in the x-ray beam. The muscle rested on a ledge which permitted application of fluid and facilitated consecutive exposures of different parts of the same muscle. Electrical connections inside the x-ray diffraction camera made it possible to stimulate the specimen electrically during the x-ray exposure. The correct position of the muscle with respect to the center of the x-ray beam was observed through a special self-illuminating microscopical device. In each side of the camera was a small hole in the horizontal axis through which threads could be attached to the ends of the muscle thus making it possible to apply tension to the muscle. Dried muscles were studied either in the usual cameras or in the special camera using an object-film distance of 40 mm. The dried muscles were fixed with plasticene across the centered hole of a glass slide.

The x-ray diffraction patterns were first studied on a viewing box. In order to compare to better advantage patterns obtained at different object-film distances, a photographic enlarger was used. This method was especially successful in making exact measurements because necessary pencil marks could be made without marring the original film.

All experiments were made on sartorius muscle of *Rana pipiens*.<sup>1</sup> The fresh muscles were kept in Ringer's solution until used and were moistened during the exposure with Ringer's or the solution whose effects were being studied. Drying of the muscles was done at room temperature in a vacuum desiccator over calcium chloride.

In the first series of experiments, the x-ray diffraction patterns of living muscle were studied under the special conditions necessitated by the exposure itself. The dimensions of our camera made short exposures (no more than 3 to 6 minutes) possible. Nevertheless, the influence of 30 minute exposures was studied. The muscle was, of course, kept moist with Ringer's solution. It could be shown that after such an irradiation, corresponding to 40,830 roentgens, the muscle was still able to respond to electrical stimulation in a manner comparable to that of a normal muscle. Nor did the histological picture reveal any gross changes. The x-ray diffraction pattern of such a muscle was identical (save for the intensity of the lines) with the ones produced by exposures of 3, 6, and 12 minutes. These findings are somewhat at variance with reports of Boehm (1931) who described the detrimental effects of x-rays on the muscle as indicated by currents of injury. Therefore, he moved the muscle continuously during the exposure. We noticed, on the contrary, that mechanical pulling of the muscle induces contraction in the latter. But part of the discrepancy between our results and those of Boehm may be explained by the fact that for these particular experiments he used a distance from the specimen to the focal spot of only 18 mm., which resulted in an irradiation of 85,000 r in 15 seconds.

<sup>1</sup> Thyrohyoid muscle of the cat was used in a few instances.

In agreement with the observations of other authors, it could be shown that the x-ray diffraction pattern of fresh muscle is to a large degree dependent upon the state of contraction of the muscle. In the experiments to be reported here a number of agents were used which induce contractions in muscle. Therefore, rigid frames of either glass or stainless steel were used. Muscles were tied to these at their ends in such a manner as to prevent contraction. In order to obtain data comparable with those reported before, several exposures were taken during which the muscle was allowed to contract against the pull of a weight.

The x-ray diffraction patterns of living sartorius muscles have been taken in thirteen instances. According to our findings, the pattern consists in (1) a spacing corresponding to  $9.57 \text{ \AA.u.}$  which is oriented perpendicular to the long axis of the muscle fibers. The end points of this streak-like blackening in the diffraction pattern lie on an ellipse which is very faintly indicated; (2) two sickles, the centers of which lie on a line parallel to the long axis of the muscle fiber. They correspond to a spacing of  $5.52 \text{ \AA.u.}$  They are possibly parts of a flattened ellipse, although in normal muscles the poles of the long axis of the ellipse could not be seen on the film. This may be partly due to the existence of (3) a diffuse, intense ring corresponding to a spacing of  $3.5 \text{ \AA.u.}$  (Fig. 1). If the living muscle is allowed to shorten before or during the exposure (as upon mechanical or electric stimulation), all of the diffraction pattern disappears excepting the diffuse ring. If such a contraction (see stimulation by single electric shock) is prevented by tying the muscle in a rigid frame, the original orientation reappears.

In order to study a transverse section, the fresh thyrohyoid muscle of a cat was tied to a frame and kept for 24 hours in 3.7 per cent formaldehyde. Thereafter a transverse section was made: the rest of the muscle was used as a control, in a longitudinal section. The pattern of the transverse section consists of two diffuse rings and of a "halo." The rings, corresponding to spacings of  $4.53$  and  $9.66 \text{ \AA.u.}$ , do not show any orientation. The diffraction pattern of the muscle in longitudinal position shows equatorial streaks and sickles, corresponding to similar spacings in the pattern of frog muscle. The distinctness of the pattern is somewhat impaired by the formaldehyde treatment (see below), but the pattern is still recognizable.

In order to obtain muscle x-ray diffraction patterns comparable to those of dried proteins, the fresh muscles were dried in a desiccator, at room temperature, to constant weight. Shrinkage in two dimensions was prevented by adhesion of the muscle to a glass surface. The loss in water after such drying amounted to about 80 per cent of the original weight.

In a preliminary experiment, a dried muscle was finely ground and the powder studied in the usual way. The x-ray diffraction pattern of such a powder is identical with the usual picture of undenatured protein (Fig. 1). It consists

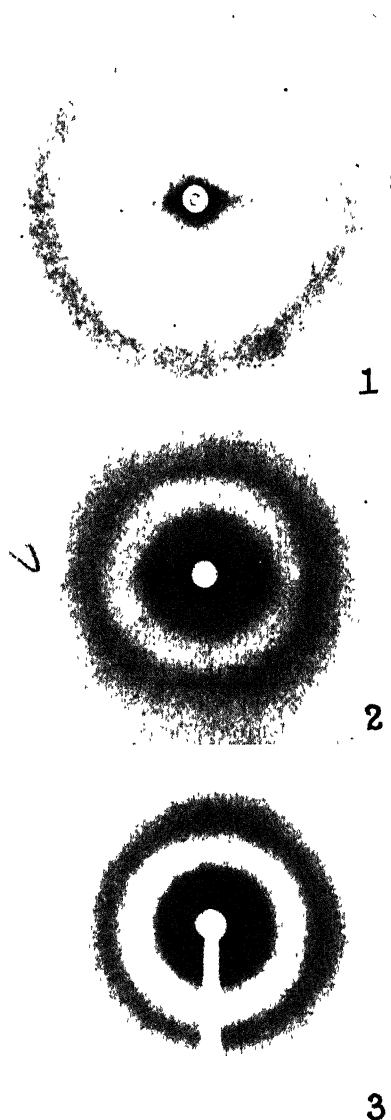


FIG. 1. Normal muscle. No. 1, fresh normal muscle. No. 2, dried normal muscle. No. 3, powdered dried muscle. In these figures, as in those that follow, the axis of the specimens is vertical. Unfortunately the two meridional sickles were not brought out in the reproduction.

of two diffuse concentric rings corresponding to spacings of 4.46 and 9.66 Å.u. All the other studies were made on whole dried muscle. The x-ray diffraction pattern of a transverse section of dried thyrohyoid cat muscle is practically

identical with the x-ray diffraction pattern of powdered muscle, whereas the control part of the muscle, exposed in longitudinal direction, shows orientation of the pattern.

The x-ray diffraction pattern as derived from a study of twenty-eight different dried frog muscles consists of (1) two equatorial points lying on an elliptical ring corresponding to a spacing of 9.95 Å.u. and of (2) an elliptical ring, flattened at its vertical pole, the diameters of which correspond to spacings of 5.11 and 4.60 Å.u. (Fig. 1). A comparison of these figures with the measurements of fresh muscles gives the following results: With the exception of some small numerical differences, the significance of which will be discussed presently, there seems to be a close similarity between the patterns of the moist and of the dried muscle. Further evidence of this similarity is given by x-ray diffraction studies of muscles in various stages of desiccation. A comparison of these two pictures with those of the moist and dried specimens shows the gradual changes of the longitudinal streaks of the moist pattern into the equatorial dots of the dried one, the increasing accentuation of the sickles and their changes to an elliptical ring, and the final disappearance of the water halo. It seems probable, therefore, that the loss of the bulk of water which occurs upon drying does not materially affect the molecular structure of the muscle. Conversely, small differences seem to exist between the horizontal diameters of the inner rings of the moist and dried muscles on one hand and the vertical diameters of the outer rings on the other hand. Since the question of the location of the water in the muscles seems to depend upon the identity of the diffraction lines of muscles in the moist and dry state, diffraction patterns of thirteen normal moist muscles were compared with those of twenty-eight normal dried muscles. The average values are summarized in Table I.

A study of these results shows that the magnitude of the differences for the diameters of the inner and the outer rings is not identical; nor are the differences in the same direction. In order to ascertain if these differences have a certain meaning or are caused by the spreading of the individual values, statistical treatment of the results was tried. According to computations based on a comparison of the two sets of means by calculation of  $t$  in order to determine  $P$  (the probability integral), both differences are significant, although to a slightly different degree: differences in the diameter of the outer ring in the moist and dried muscles cannot occur by chance in 1 in 100 cases, while there are 1 to 2 chances in 100 that the differences observed in the diameter of the inner ring are not significant.

In order to check this point more carefully and to exclude the possibility that the differences were due to variations in the thickness of the muscles, a more rigorous experiment was carried out. In nine cases x-ray diffraction patterns were made of normal moist (living) muscle; the muscles were then dried and another set of x-ray diffraction patterns made. A comparison of the

measurements shows that the average figures are identical with the ones obtained in the larger series (Table II). But a statistical evaluation of these

TABLE I  
*Figures on 13 Moist and 28 Dried Muscles Corrected to 40 Mm.*

Moist muscles				Dried muscles				
Film No.	Inner points	Outer sickles	Water ring	Film No.	Inner ring		Outer ring	
					Horizon- tal	Vertical	Horizon- tal	Vertical
	mm	mm.	mm.		mm.	mm.	mm.	mm.
(1) 14 C	13	23.5	38 × 38	(1) II	13	12	28	26
(2) 20 C	13.5	23	39 × 38	(2) III	12.5	12	28	25.5
(3) 22 C	12	21	35 × 36	(3) IV	13	12	28.5	25.5
(4) 27 C	12.5	24.5	38 × 38	(4) V	13	12	28	25.5
(5) 37 C	12.5	21.5	36 × 36	(5) VI	13	12	28.5	25.5
(6) 40 C	15	23	38 × 38	(6) VII	13.5	12.5	28.5	25.5
(7) 69 C	13.5	24.5	40 × 39	(7) VIII	12.5	12	28.5	25.5
(8) 167 C	13	23	38 × 38	(8) XI	12.5	11.5	28	25
(9) 168 C	14	23	39 × 39	(9) XVI	12.5	11.5	28	24.5
(10) 169 C	13	23	38 × 38	(10) XVIII	12.5	11.5	28.5	25.5
(11) 195 C	13	24	39 × 39	(11) 2 C	13	12	28.5	24.5
(12) 198 C	13	24	39 × 39	(12) 4 R	12.5	12	28.5	25.5
(13) 199 C	13	23	38 × 38	(13) 5 R	12.5	12	28	25
				(14) 9 R	12.5	11.5	28	25
Total . . .	171	301		(15) 10 R	12.5	12	28.5	25
				(16) 58 R	12.5	12	28	25.5
Average ..	13.1	23.1		(17) 70 R	12.5	12	28	26
	13	23		(18) 75 R	12.5	11.5	28	24.5
				(19) 78 R	12.5	11.5	27	24.5
				(20) 21 R	12.5	12	28	25.5
				(21) 27 R	12.5	12	27	25
				(22) 29 R	12.5	12	27	25
				(23) 138a R	13.5	11.5	28	25
				(24) 142 R	13.5	11.5	28	25
				(25) 175 C	13	12	28.5	24.5
				(26) 163 R	12	11	28	25
				(27) 204 C	13	12	28	24.5
				(28) 164 R	12.5	11.5	28	25
				Total. . .	356.0	331.0	785.5	704.0
				Average . . .	12.7	11.8	28.05	25.1
					12.5	12	28	25

results made to determine the significance of the mean of a unique sample (Fisher, 1930), indicates that only the differences observed in the vertical diameter of the outside ring in moist and dried muscles are significant; *i.e.*, there is less than 1 chance in 100 that a deviation as large as the ones observed

might occur accidentally. We do not quite feel able to decide whether the results obtained with the larger series are better or worse than the ones achieved with the smaller but more select series. But, in either case, our results on the influence of drying upon the spacing corresponding to the horizontal diameter of the inside ring are different from those reported by Boehm (1931). This author reports, on the one hand, an increase in the spacing determining the distance of the individual myosin chains from each other when the dried muscles take up water. (According to Boehm the change is from 10–11 Å.u. to 11 Å.u.). Boehm does not give the number of observations on which he bases his conclusions nor the exact methods used for the drying of muscles. Our results

TABLE II  
*Figures on Same Muscle—Living and Dried Corrected to 40 Mm.*

Moist muscle				Dried muscle					
Film No.	Horizon- tal inner ring	Vertical outer ring	Water ring		Film No.	Inner diameter		Outer diameter	
	mm.	mm.	mm.	mm.		Horizon- tal	Vertical	Horizon- tal	Vertical
(1) 167 C	13	23	38 × 38	138a R	13	11.3	28	25.5	
(2) 168 C	14	23	39 × 39	142 R	13	11.5	28	24.5	
(3) 169 C	13	23	38 × 38	175 C	12.5	11.5	28	24.5	
(4) 195 C	13	24	39 × 39	163 R	12	11	28	25	
(5) 198 C	13	24	39 × 39	204 C	13	12	28	24.5	
(6) 199 C	13	23	38 × 38	164 R	12.5	11.5	28	25	
(7) 27 C	13	24	36 × 36	21 R	12.5	12	28	25.5	
(8) 37 C	13	22	36 × 36	27 R	12.5	12	27.5	25	
(9) 40 C	14	23	37 × 37	29 R	12.5	12	28	25	
Total . . . . .	119	209	340 340	Total	113.5	104.8	251.5	224.5	
Average .	13	23	38 38	Average	12.5	11.5	28	25	

were not modified further by heating the dried muscle for 2 hours at 105°C. with respect to either the x-ray diffraction pattern or the water content.

On the other hand, Boehm does not find any differences of the elliptical (outside) ring in moist and dried muscles. We have tried to interpret the differences in the vertical diameter of the outside rings of moist and dried muscle. Astbury and Bell (1941) have correlated the corresponding spacings to the length of longitudinal folds in the myosin chains. Our results seem to indicate that a small amount of water, causing a change of spacing from 5.52 Å.u. to 5.11 Å.u. in this dimension, is lost from the moist muscle upon drying. Conversely, if a reversibility of this process is taken for granted, it would mean that water taken up by the muscle increases the length of the longitudinal folds. Since an increase in the longitudinal folds should cause a corresponding increase



in the total thickness of the muscle, there is no real discrepancy between our results and the known fact that muscles increase in thickness only upon uptake of water. According to earlier observations (Embden, 1925) which we were able to confirm, frog muscles contain water up to 80 to 90 per cent of their total weight. Our results confirm the findings of Boehm inasmuch as we too must assume that most of this water is intermolecularly located.

Another experiment was made in order to study the nature of the muscle changes upon drying. An x-ray exposure was made of a living moist muscle: the muscle was dried and again an exposure was made. Finally the dried

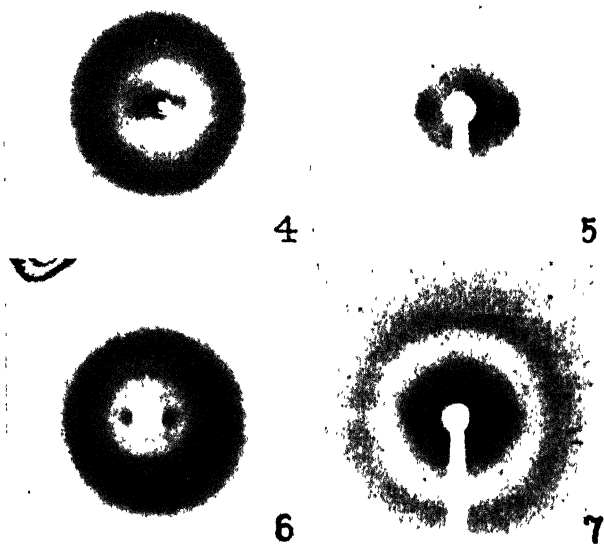


FIG. 2. Effects of drying and soaking. No. 4, fresh muscle. No. 5, same muscle dried. No. 6, same muscle res soaked. No. 7, same muscle redried.

muscle was res soaked in Ringer's solution (Fig. 2). Microscopically it looked very much like the original moist muscle, but, of course, did not show any response upon electrical stimulation. Its x-ray diffraction pattern was practically identical with the one observed in the same muscle when still alive. These findings seem to add additional evidence to the theory of the identity of the diffraction pattern in moist and dry muscle. It shows besides that whether the muscle is alive or not does not modify its fine structure. This supports the assumption that the x-ray diffraction pattern of the muscle is essentially the one of myosin. Finally it indicates that the dehydration of the muscle, as done by our method, is a reversible process as far as the fine structure of the muscle is concerned.

The importance of stretching in the production of orientation and in the increase or change in the orientation, in fibers of homogeneous material has been demonstrated by Katz and Astbury. Therefore, experiments were started in this direction. The muscles were stretched by different weights and

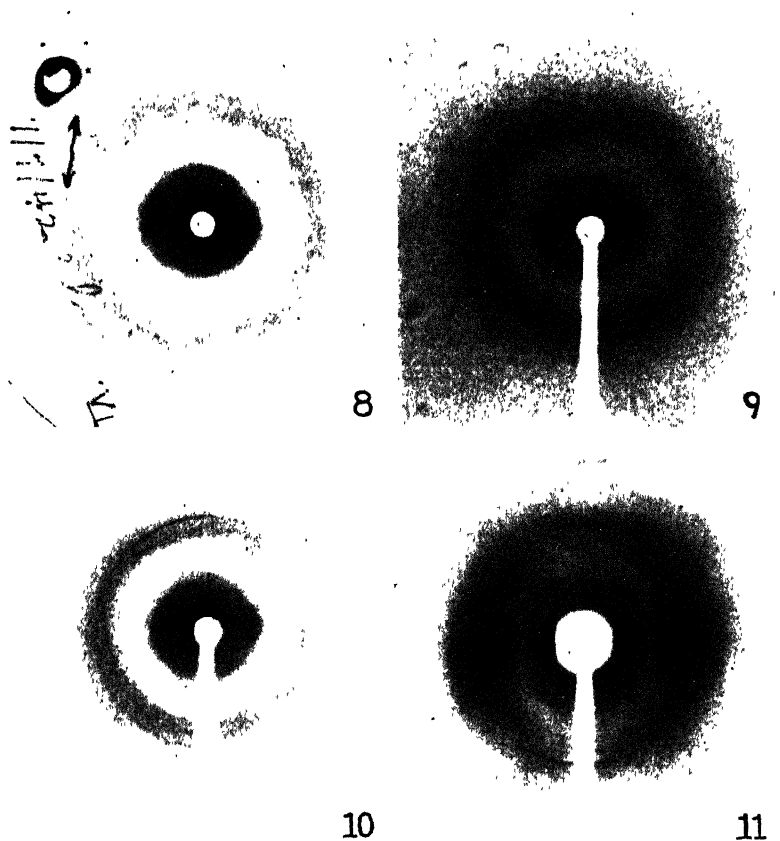


FIG. 3. Effects of stretching. No. 8, muscle stretched, 5 gm. No. 9, muscle stretched, 42.5 gm. No. 10, muscle stretched, 70.0 gm. No. 11, frog tendon, stretched, 70.0 gm. All specimens after drying.

allowed to dry in a desiccator at room temperature. Preliminary experiments showed that the tearing point of the muscle was reached with 100 gm. Therefore, 5 to 70 gm. were used (Fig. 3).

The x-ray diffraction patterns show the following results: Muscles stretched with 5 or 20 gm. weights did not show any particular changes of the diffraction pattern. Applying a weight of 42 gm. results in the appearance of a faint line outside the sickle; this line corresponds roughly to a spacing of  $4.32 \text{ \AA.u.}$

Stretching by 70.3 gm. makes this line more distinct, its spacing remaining the same: the whole ring becomes visible, although the stronger intensity in the parts parallel to the horizontal axis of the diffraction pattern indicates a distinct orientation.

In order to identify this ring analogous experiments were made on the Achilles tendon of the frog. Unstretched tendon does not show any ring corresponding to a spacing of 4.32 Å.u. Upon stretching with 70.3 gm. such a ring appears, showing a degree of orientation similar to the one described in stretched muscle (Fig. 3). The implication seems obvious: the ring visible upon stretching is due to a substance common to muscle and tendon. Herzog and Jancke (1926) have described additional lines and sickles that become visible in the x-ray diffraction patterns upon stretching. Some of them have close topical relations to the elliptical ring similar to the ring which we have described. For some reason Boehm was unable to increase the number of diffraction lines by drying and stretching.

According to earlier work, temperatures up to 36°C., if lasting only a short time, produce a reversible contracture of the muscle. Exposure of muscle to a temperature of 50°C. for only a few minutes produces irreversible changes, including a loss of elasticity of the muscle. Higher temperatures induce further contraction, as can be observed in any dead tissue (Riesser, 1925).

In order to study the influence of higher temperatures upon the x-ray diffraction pattern of striated muscle, sartorius muscles were immersed for 5 to 30 minutes in Ringer's solution as well as isotonic dextrose solutions at 35 to 50° and 100°C. In order to prevent contracture the muscles were tied in frames. X-ray diffraction patterns of these muscles were taken before and after drying. No changes could be detected either in the moist or in the dry specimen that had been kept at 35°C. Nor was the response to electrical stimulation affected. The muscles kept at 50°C. for 5 minutes show in the diffraction pattern of the moist specimen only very faint signs of the inside equatorial orientation. The outside sickles have disappeared completely. The diameter of the diffuse ring remains unchanged. The dried specimens show only traces of the equatorial points which are very much elongated and the ring on which they lie is much darker than usual. The outside ring is sharper than in the pattern of the normal specimen and the flattening in the vertical axis has disappeared.

After 5 minutes' immersion in boiling Ringer's or dextrose solution, the wet specimen shows a complete loss of equatorial orientation. In place of the sickles a ring of even density appears, the diameter of which corresponds to a spacing of 5.3 to 5.5 Å.u. The diffuse water ring does not show any changes. The dried specimen is characterized by a uniform inside ring, without equatorial points. The outside ring is a circle which is very markedly sharpened. The diffraction pattern resembles closely the picture described in heat-denatured proteins (Fig. 4 and Spiegel-Adolf and Henny, 1941).

Because of reports in the literature that muscles submitted to the influence of heat ( $50^{\circ}\text{C}.$ ) stretch more easily than unheated muscle, some experiments were made on this point. Heated muscle tears when weighted with only 30 gm.

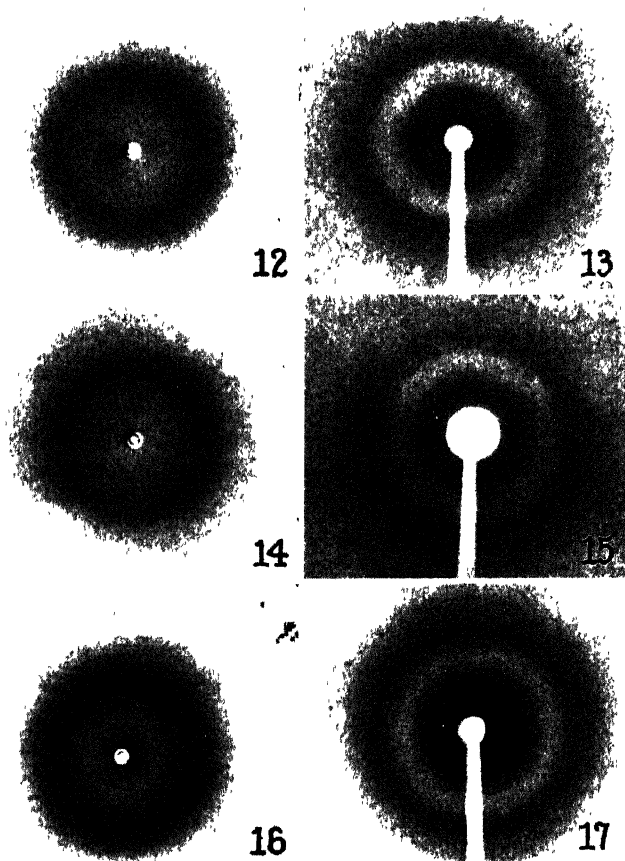


FIG. 4. Effects of heat. Nos. 12 and 13, muscle, 30 minutes at  $35^{\circ}$ . Nos. 14 and 15, muscle, 5 minutes at  $50^{\circ}$ . Nos. 16 and 17, muscle, 5 minutes at  $100^{\circ}$ . In Figs. 4 and 6 to 9 the left photograph of each pair refers to the moist specimen, the right photograph to the dried one.

When 20 gm. were used a marked elongation of the muscle resulted. Nevertheless, no orientation in the diffraction pattern of the heated muscle was observed.

The effects of ultraviolet radiation upon protein have been extensively studied and compared in their effects with changes brought forth by heat and  $\gamma$ -rays (Spiegel-Adolf 1927, 1928). It seemed interesting, therefore, to study

the influence of ultraviolet light upon the x-ray diffraction of irradiated muscle. Effects of ultraviolet light upon muscle have been described by Azuma and Hill (1927), Lippay (1929), and Ashkenaz (1938). In our experiments we used a Hanovia quartz mercury lamp operating on direct current at 220 volts. The muscles were irradiated while immersed in Ringer's solution in a quartz beaker. In view of the effects of heat upon the x-ray diffraction patterns of muscle, care was taken that the temperature of the muscle did not exceed room temperature. This was done in one set of experiments by working at a distance of 20 inches from the lamp. In another series of experiments, conducted at a distance of 10 inches the beaker was packed in ice. The irradiation time was varied between 15 and 120 minutes, during which the muscle was repeatedly turned over. The muscles were irradiated either under isotonic conditions, or they were weighted or tied in frames. X-ray diffraction patterns were taken both from the moist irradiated samples and from the same muscles after drying. The response to electric stimulation was tested in all moist samples. The results of nine experiments can be summarized in the following way:

1. Muscles allowed to shorten isotonicly under irradiation show changes in the x-ray diffraction pattern after a 15 minute exposure to the mercury quartz lamp at 20 inches from the arc. These changes are not visible in the pattern of the moist sample but show up in that of the dried one, as an elongation of the orientation points. After an exposure of 90 minutes, the muscle no longer responds to electric stimulation, and the diffraction pattern of the moist sample shows that the equatorial streaks have very nearly disappeared; in the corresponding dry samples, the equatorial points are elongated, the sickles have disappeared although the outside ring is still elliptical. If the muscle has been allowed to stand in Ringer's solution overnight in the refrigerator, then two outside rings of diameters corresponding to 2.88 and 2.02 Å.u. appear, the intensity of which decreases in the above sequence.

2. Muscle weighted with 1.5 gm. or tied in a frame and irradiated at 20 inches from the arc for 30 minutes does not show any appreciable changes in the diffraction pattern of either the moist or dried sample.

3. Such changes start to become manifest in muscle tied in frames upon an increase of the irradiation time to 120 minutes. An irradiation of 90 minutes produces diffraction patterns nearly identical with the ones observed in isotonicly shortened muscles irradiated for the same length of time. When the distance from the arc is decreased to 10 inches, an irradiation of 90 minutes produces a nearly complete destruction of the orientation. But although the equatorial points as well as the outside arcs are very faint in the dried sample, nevertheless, the elliptical shape of the outside ring and the more or less diffuse character of the latter are preserved. Two additional rings become visible upon standing of the sample in Ringer's solution overnight in the refrigerator. Their spacings correspond to 2.85 and 2.01 Å.u. These rings can be identi-

fied with the diffraction pattern of NaCl. They are visible even if the muscle has been wiped off with filter paper before drying. The existence of these salt rings is tentatively explained by an increase of the permeability of the muscle cells, which permits the NaCl of the Ringer solution to penetrate into the interior of the cells. We found and shall discuss similar results in later experiments which involve more or less complete loss of orientation in the x-ray diffraction patterns. This relation is not necessarily reversible as shown by the patterns of boiled muscles (see below).

A comparison of the effects of heat and of ultraviolet light on the x-ray diffraction pattern of muscle clearly indicates the differences. It is interesting that some of these differences are analogous to those observed in proteins subjected to similar treatment (Spiegel-Adolf and Henny, 1941). Both in proteins and in muscle heat denaturation produces a sharpening of the backbone reflection. And in both these changes are absent upon intense ultraviolet irradiation. These findings corroborate the assumption that the x-ray diffraction pattern of the muscle is fundamentally that of myosin.

The ultraviolet radiation used on the muscle was more than sufficient to coagulate and denature proteins (Spiegel-Adolf, 1927). There is not definite proof that heat and ultraviolet light denaturation are quantitatively comparable. Nevertheless, it must be stressed that the loss of orientation is complete in heat denaturation while even our most intense ultraviolet irradiation was unable to destroy the elliptical configuration of the backbone spacing. Besides, although the equatorial points are lost, there is no trace of the inside ring corresponding to the spacing of 21.3 Å.u., which appears in the heat-denatured muscle. Although these findings seem to point to the greater extent of the changes in boiled muscles, the latter do not show salt rings, even when allowed to soak in cold Ringer's solution for 12 hours after boiling. This may mean that loss of orientation is not necessarily correlated to changes in permeability or that heat coagulation in itself causes a compensating increase in density of the cell membranes.

The influence of electrical stimulation upon the x-ray diffraction pattern of muscle has been studied by Boehm (1931). We have tried to reduplicate his findings and come to a clearer understanding of the basic mechanism. For this purpose we tried two different lines of approach.

In the first series, we stimulated the muscle faradically, using a standard Harvard inductorium in which the primary current was provided by a 4.4 volt storage battery. Coil distances of 0 to 10 cm. were used. In preliminary experiments the movements of sartorius muscles of the frog under faradic stimulation were recorded with a kymograph. It could be shown that contraction upon stimulation was only maintained for 3.1 minutes and that at least 30 gm. of weight attached to the muscle were necessary in order to prevent shortening of the muscle. Experiments were, therefore, made in which muscles were

stimulated during the x-ray exposure. It was mentioned previously that the inside of our cameras is provided with electrical connections. Cotton threads soaked in Ringer's solution were used as electrodes. Two periods of stimulation totaling 6 minutes were used in one exposure. The muscles were loosely tied in a frame so that contraction was easily visible when the irritability of the muscle was tested after exposure.

In another set of experiments the muscle was permitted to contract isotonically under a weight of 30 gm. while faradically stimulated as described above. This was accomplished by tying one end of the muscle to a fixed point while the other end was attached to a thread which moved freely through a hole in the horizontal axis of the camera, over a pulley, and supported a 30 gm. weight.

In both sets of experiments the muscle still reacted to electrical stimulation after the exposure had been completed. A comparison of the x-ray diffraction patterns gives the following results: In the case of the muscle which was free to contract, the orientation of the x-ray diffraction pattern was lost. In the muscle in which the shortening had been largely prevented by the weight, the orientation was practically normal. It seemed worthwhile to put these findings on a wider base. It was not possible to vary the frequency of the faradic stimulation, and it seemed possible that by using less frequent stimuli less fatigue of the muscle and therefore, a longer period of contraction, could be obtained. Therefore, the muscle was stimulated by 240 to 360 single induction shocks per minute by manual operation of the switch in the primary circuit of the inductorium. The coil distance was varied from 0 to 10 cm. Muscles stimulated in this way for 6 minutes were studied under three different conditions: while tied in a frame, under isotonic conditions, or weighted with 10 to 20 gm. The x-ray diffraction patterns of muscles tightly tied in frames showed fair orientation; when the muscles were weighted, the distinctness of the orientation increased with the weight (from 10 to 20 gm.). Muscles permitted to contract isotonically lost their orientation either completely or showed only faint traces of it. In an attempt to differentiate between the effects of electrical stimulation and of mechanical shortening, such contracted muscles were stretched mechanically and again a diffraction pattern taken. The orientation which had disappeared became visible again with practically the same degree of intensity as in the normal rested muscle (Fig. 5.). These observations seem to support the assumption of Boehm that the disappearance of the equatorial points upon isotonic contraction is a purely mechanical phenomenon based on deformation of the micellae. But, contrary to this author, the elliptical ring becomes circular in our experiments. Upon stretching of the muscle it reassumes its elliptical shape and the sickle-like intensities reappear. The difference between the disappearance of the equatorial points after isotonic contraction upon electrical stimulation and upon heat denaturation is quite obvious since, among other things, no amount of stretching of the heat-denatured muscle is able to restore the orientation.

There is a marked parallelism between the changes in x-ray diffraction and changes in birefringence. In his monograph, Schmidt (1937) mentions experiments of von Ebner who has observed a decrease in birefringence in living muscle upon electrical stimulation. According to von Muralt (1932), who made elaborate studies on single contractions of frog muscle, these changes in the birefringence are changes of the fine structure of the main valency chains; *i.e.*, of the substances which are supposed to give the x-ray diffraction pattern. Bozler and Cottrell (1937) came to the conclusion that the diminution of bire-

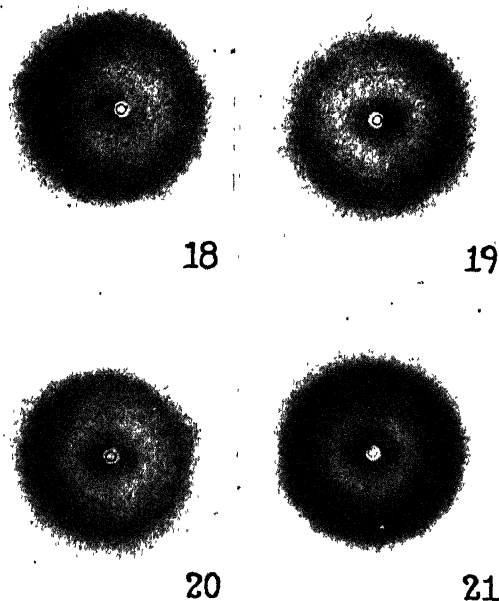


FIG. 5. Electrical stimulation. No. 18, after 1st contraction. No. 19, after 2nd contraction. No. 20, after stretching. No. 21, normal moist muscle.

fringence observed in the sartorius muscle of the frog during isotonic contraction is due to the shortening alone and not directly to the contractile process.

A number of experiments were made with the purpose of studying the influence of various neutral salts upon the x-ray diffraction pattern of muscle. The salts investigated were NaCl, KCl,  $\text{NH}_4\text{Cl}$ , LiCl, NaCNS, Na citrate,  $\text{K}_2(\text{COOH})_2$ , and  $\text{MgCl}_2$ . As in proteins it is necessary to differentiate between the effects of salt concentrations and salt ions. Two salt concentrations were used. The first one is isosmotic with 0.73 per cent NaCl, which according to Hill and Kupalov (1930) is in osmotic equilibrium with the blood salts of the frog. The second concentration is isosmotic with 10 per cent  $\text{NH}_4\text{Cl}$ .



The possibility exists that some of the salts, especially the ones with a bivalent cation, do not penetrate into the muscle cells as easily as salts with univalent ions. Therefore, a number of controls were run in which normal muscles and muscles with cut ends were used. In the majority of cases the muscles were tied in frames; in some of the earliest experiments muscles were pinned on cork. The mounted muscles were immersed in the different salt

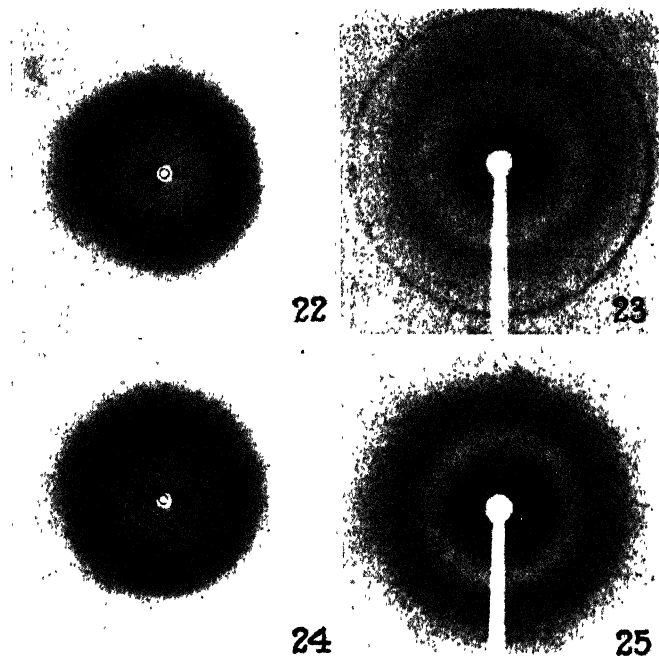


FIG. 6. Effects of KCl and NaCl. Nos. 22 and 23, muscle after 94 minutes in KCl, before and after drying. Nos. 24 and 25, muscle after 99 minutes in NaCl, before and after drying.

solutions for periods of time varying between 30 and 100 minutes. Thereafter, the muscles were rinsed with Ringer's solution and x-ray diffraction patterns were made of the moist samples. In nearly every case the specimens were then dried in the usual way and x-ray exposures were made of the dried samples. In order to identify some of the salt lines in the diffraction patterns of the salt-treated muscles, x-ray exposures of the salts in question were made. In addition, the data of Hanawalt and coworkers (1938) were used as references. The results of 174 x-ray diffraction studies may be summarized in the following way: No neutral salt with a univalent cation, when used in a concentration equivalent osmotically to 0.73 per cent NaCl, with the exception of KCl, seems to

have any effect upon the x-ray diffraction pattern. KCl, at a comparable concentration, destroys the orientation of the pattern to a marked degree inasmuch as, in the moist specimen, the equatorial streaks are weak and in the dry specimen, faint arcs replace the equatorial points. The sickles also are lost, but the elliptical shape of the outside ring is maintained. Two salt rings appear, corresponding to spacings of 3.19 and 2.28 Å.u., which are practically identical with salt rings measured in a sample of powdered KCl. The fact that KCl is

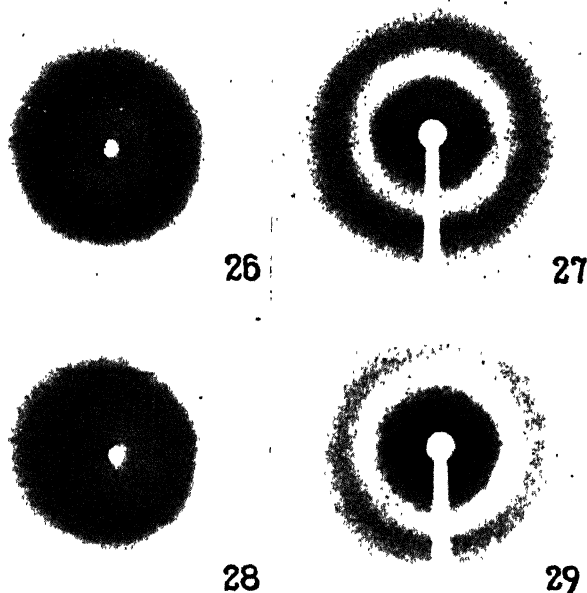


FIG. 7. Effects of  $\text{MgCl}_2$  and  $\text{CaCl}_2$ . Nos. 26 and 27, muscle exposed to  $\text{MgCl}_2$ , before and after drying. Nos. 28 and 29, muscle exposed to  $\text{CaCl}_2$ , before and after drying.

effective in a concentration in which NaCl does not produce visible changes may be due partly at least to the "loosening effects of K ions on cell membranes" (Abderhalden, 1927) (Fig. 6).

These findings are of interest since they offer a physicochemical correlation to the well known physiological differences in the effects of Na and K salts.  $\text{MgCl}_2$  in the above concentrations does not produce marked changes in the x-ray diffraction patterns of muscle. The only change, if any, seems to indicate rather a relaxation of the muscle as concluded from the very pronounced orientation marks. Higher valency of the anion, as in oxalate and citrate, apparently does not increase the effect in most of the salts (Fig. 7).

$\text{CaCl}_2$ , at the same concentration produces marked changes in muscles with

cut ends and in normal muscles, when the immersion time is increased to longer periods. In extreme cases, the orientation seems to disappear to a large extent, the outer elliptic ring in the sample loses its sickles and polar flattening, and salt rings corresponding to spacings of 2.85 and 2.01 Å.u. become visible. This happened only in a muscle which after immersion in  $\text{CaCl}_2$  was bathed for an hour in Ringer's solution. This can be interpreted as an expression of the antagonism existing between the increased permeability which we have frequently observed upon loss of orientation and the known membrane-tightening effects of Ca salts. The salt rings observed correspond in their spacings to the NaCl pattern (Fig. 7).

The effects of hypertonic salt concentrations studied with NaCl, NaCNS,  $\text{NH}_4\text{Cl}$ , and  $\text{MgCl}_2$ , show similar results. In every case, equatorial points and sickles disappear and the elliptical outer ring has changed to a circular shape. Salt rings corresponding to the spacings characteristic of the salts used are clearly visible. These findings are in agreement with similar observations of Worschitz and von Hermann (1934), who point out the irreversibility of the changes in the x-ray diffraction patterns after removal of the hypertonic salts by washing. Von Ebner (1882), working with hypertonic NaCl, and Biedermann (1927), who tested the influence of 10 per cent  $\text{NH}_4\text{Cl}$  upon muscles of frogs, reported marked decreases in birefringence.

NaCNS has effects which are widely different. Not only does the orientation disappear but the outside ring gets wider in diameter and more diffuse. It is slightly elliptical, but the longer diameter is in the vertical axis. Salts have apparently penetrated into the muscle cells, but the salt arcs are decidedly oriented as if the salt were crystallized under stress. It is a well known fact that thiocyanates have a hydrating effect upon proteins. In our studies on acid (see below) we have found a similar effect upon the width of the outside rings, and we shall try to explain this phenomenon. The orientation apparent in the salt rings may be related to the hygroscopic character of the salt, which influences its time of crystallization so as to coincide with changes in the muscle upon drying.

$\text{LiCl}$  in hypertonic concentrations also destroys the orientation of the muscle diffraction pattern. Also there are indications of a very faint ring with the spacing at 21.3 Å.u. similar to rings observed both in heat-denatured and  $\text{HNO}_3$ -treated muscles. No explanation is offered for this observation, but Worschitz (1934) has observed a spacing of 22 Å.u. in dried muscle, which we have been unable to confirm in normal material. In addition, we have observed a similar diffraction ring in a sample of powdered muscle nuclei. Another special feature of the  $\text{LiCl}$  is a splitting up of the outer circular ring. The diameter of the smaller ring corresponds to a spacing of 4.70 Å.u., of the larger ring, to 3.77 Å.u. The space between these two rings is diffusely blackened. The larger ring has no equivalent in the  $\text{LiCl}$  diffraction spectrum.

$\text{CaCl}_2$ , in hypertonic solution, prevents the recording of an x-ray diffraction

pattern of muscle. Apparently Ca ions penetrate through the cell membranes since the muscle becomes relatively opaque to x-rays. The inner ring of the dried muscle pattern is just barely visible.

The influence of acids upon muscles has been studied extensively, especially with respect to changes in birefringence and swelling. Since muscles produce acid as a part of their particular metabolism, such studies have some immediate bearing upon certain physiological and pathological problems of muscle. Furthermore, the numerous physicochemical analyses of reactions between proteins and acids promise further help in the elucidation of analogous problems in muscles.

We have studied the effects of HCl, HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>, CH<sub>3</sub>COOH, moniodoacetic acid, and lactic acid upon the x-ray diffraction of muscles. The concentrations of the acids varied between 0.0005 N (moniodoacetic acid) and 1.7 N (acetic acid). The solutions were made isosmotic when necessary by adding the required amounts of Ringer's solution or dextrose. All the muscles immersed in acid solutions were tied in frames. The time of immersion varied between 10 minutes and 28 days. X-ray diffraction patterns were made from the moist muscles and also after drying.

A study of 63 different x-ray diffraction patterns led to the following conclusions. Corresponding to visible swelling following acid treatment, most of the moist muscles show a slight increase in diameter of the diffraction ring corresponding to a slight decrease in interplanar spacing. In the case of HCl, systematic studies were made to determine the influence of acid concentration and duration of immersion in the acid, upon the diameter of the water ring. Variation in HCl concentration did not give a clear cut result. But, if the HCl concentration is kept at the same value (0.1 N) and the duration of immersion varied between 30 and 80 minutes, then the diameter of the water ring seemed to pass through a minimum at 45 minutes before reaching a practically constant value. These findings show a certain similarity with the reactions of proteins and acids. The viscosity graph of globulins and HCl also passes through a peak and the viscosity drops again within the 1st hour (Spiegel-Adolf, 1923).

Although the water ring in muscles is not supposed to be a specific diffraction line, yet it may indicate different degrees of swelling of the muscle tissue. The effect of the acids upon the x-ray diffraction pattern of muscle seems to depend largely upon the pH of the solution. The mineral acids as well as lactic acid in concentrations up to 0.005 N apparently leave the structure of the muscle unchanged. However, the orientation seems to be more pronounced than in normal living muscle. The acid-treated muscle does not respond to stimulation and is probably dead. Further experiments may have to be performed to decide whether the shift of K ions reported in the first stages of acid poisoning of muscle or differences in tonus between living and dead muscle are responsible for this change in the x-ray diffraction pattern.

If the concentration of the acid is raised to 0.01 N or higher, then the orienta-

tion of the x-ray diffraction pattern of muscle disappears completely. In dried muscles which have been immersed in HCl, HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>, or lactic acid, the outer ring is much sharper than usual, similar to the sharpening described in muscles exposed to temperatures of 50°C. and above. Since the protein-denaturing effects of strong acids are well known, the phenomena observed may be explained in a manner similar to that offered for the effects of heat.

We have ascertained that acid denaturation in muscle is not reversible upon neutralization of the medium. In samples immersed in mixtures of acid and Ringer's solution there appears, upon drying, the characteristic pattern of NaCl. These rings are absent in patterns obtained in control experiments in which Ringer's solution was replaced by isotonic dextrose solution. This confirms the explanation previously given for the salt rings. Therefore, the appearance of the latter seems to be an additional proof of the detrimental effects of acid upon muscle. Due probably to the weakness of acetic acid, a 0.01 N solution is without apparent effect upon the x-ray diffraction pattern of muscle. With a much higher concentration of acetic acid (1.7 N) orientation is completely and irreversibly lost. The pattern resembles that of boiled muscle.

In view of references in the literature to the inhibiting effect of monoiodoacetic acid on the lactic acid production of muscle, some experiments using lactic acid were made. According to Riesser's review (1925), lactic acid takes part in the swelling of the muscle produced by heating over 49°C. However, Weber (1934) was unable to observe further coagulation by lactic acid and our own theory of heat denaturation of proteins does not require acid formation. We were able to ascertain that the characteristic changes in the x-ray diffraction pattern of muscle produced by boiling are not influenced by a pretreatment with 0.01 per cent monoiodoacetic acid up to 405 minutes immersion. Monoiodoacetic acid of this concentration does not affect the x-ray diffraction pattern of muscle. In another series of experiments we tested the effects of single electric shocks upon muscles which had been exposed for 10 to 38 minutes to 0.01 per cent monoiodoacetic acid. The muscles were either stretched by pulling on the ends or were tied on frames. Although electrical stimulation is supposed to increase lactic acid formation the diffraction patterns in the above series were not different from the ones observed in normal muscle upon electrical stimulation.

Rigor mortis according to the current opinion is "a disturbance of the colloid equilibrium of the muscle proteins produced through anoxia and conducive to maximal acid formation in the muscle" (Riesser, 1925). Therefore, a description of some of our x-ray diffraction patterns pertaining to that condition may well follow the discussion of the effects of externally applied acids. We had some technical difficulties in producing rigor mortis in frog muscle. If the muscle was removed 22 hours after the pithing of the frog, neither the x-ray diffraction pattern of the moist nor that of the dried specimen showed any

appreciable anomalies. There are indications in the literature that rigor mortis is more easily produced in fatigued muscles. Therefore, a number of muscles were electrically stimulated for 6 minutes, while tied in frames, and then kept in Ringer's solution for 21 to 24 hours. Another group was treated for a similar length of time with 0.01 to 0.06 per cent caffeine in Ringer's solution. Neither group responded to further stimulation. In the electrically stimulated muscles as well as in the caffeine treated ones, NaCl rings appeared after drying, indicating a change in permeability of the muscles. There is practically no orientation in the x-ray diffraction pattern of the caffeine-treated muscles and, in one instance, the spacing corresponding to 21.2 Å.u. became visible. In the electrically stimulated muscles, orientation persists although the points in the x-ray diffraction pattern of the dried specimens have the shape of arcs. The elliptical character of the outside ring is well preserved. These results are in agreement with those reported by Stübel and Liang (1928), who report a loss in birefringence of varying degree in different kinds of contractures.

According to Vlès (1911), KOH and  $\text{NH}_3$  destroy the birefringence of muscles. Only a few experiments were made in this direction. We could ascertain that the effective concentration of NaOH is markedly higher than the corresponding concentration of strong acids which obliterates the orientation of the muscle x-ray diffraction pattern. NaOH, in a concentration of 0.01 N, either in Ringer's solution or in dextrose, does not affect the orientation of the dried muscle, although a certain amount of swelling is indicated in the pattern of the moist muscle (faint streaks, hardly visible sickles). Higher concentrations such as were used in our experiments with  $\text{NH}_3$  (0.25 N for 40 to 86 minutes), destroy the orientation of the x-ray diffraction pattern. Stretching of the  $\text{NH}_3$ -treated muscle by weights of 5 to 50 gm., while drying, does not produce visible changes of the pattern.

The reaction of proteins and formaldehyde has been studied extensively. It is generally assumed that formaldehyde is added to the terminal  $\text{NH}_2$  groups. There seem to be indications that combination with formaldehyde produces a denaturation of the protein. Since fixation in formaldehyde is widely used in the preservation of normal and pathological specimens it seemed interesting to study the influence of formaldehyde fixation upon the x-ray diffraction pattern of muscle.

Muscles were kept for 24 hours in 3.7 per cent formaldehyde solution, some tied in frames, others free. The moist preparations not only showed marked orientation but also like muscles killed with dilute acids, a more distinct pattern than the normal muscle. The tying of the muscle in a frame seems to exaggerate the flatness of the sickles.

In the dried preparations the equatorial points seem to be slightly extended into arcs. The elliptical character of the outside ring is well preserved (Fig. 8). These findings seem to resemble known differences among the various kinds of

protein denaturation. Clark and Shenk (1937) have reported that some fibered proteins other than muscle are not affected by formaldehyde unless preswollen by alkali. The fact that formaldehyde fixation modifies only slightly the x-ray diffraction pattern of muscle seems to indicate the possibility of using material preserved in formaldehyde for x-ray diffraction studies.

Among anesthetics acting upon isolated muscle the most interesting seemed to be alcohol. Its direct effect upon proteins in comparison with other agents producing denaturation has been described by one of us (Spiegel-Adolf, 1929).

We have studied the effect of alcohol upon muscle at concentrations of 5, 15, and 95 per cent. The immersion time varied from 8 to 32 minutes. Fourteen x-ray diffraction patterns of moist and dried muscles were taken. The following results were observed. An immersion in 5 per cent alcohol-Ringer

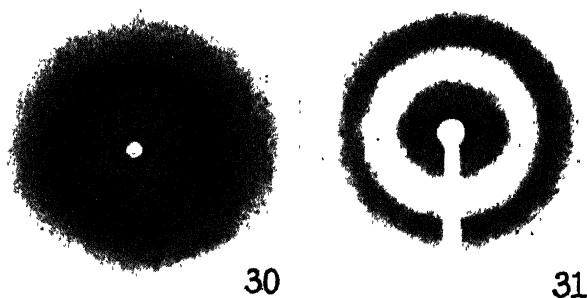


FIG. 8. Effects of formaldehyde. Nos. 30 and 31, moist and dried muscle.

(for 42 to 82 minutes), although without an anesthetizing effect upon the muscle, diminishes in the moist samples the sharpness of the streaks and the sickles disappear altogether. This behavior can possibly be explained by a stimulating effect of the low alcohol concentration. In the dried preparation scarcely any changes corresponding to the ones noticed in the moist sample can be detected. Apparently they have reverted to normal upon the evaporation of the alcohol while drying.

Muscles treated with 15 per cent alcohol are anesthetized. The streaks and sickles in the moist specimen are well defined. The corresponding picture of the dried muscle is practically normal. All muscles treated with 95 per cent alcohol show a marked decrease in the diameter of the water ring. The usual diameter of 18 to 19 mm. comes down as low as 15 mm. We have not yet an adequate explanation for this phenomenon, although we have observed a similar shrinkage in the diameter of the water ring which occurs upon treatment with acid, and according to Riesser (1925) lactic acid concentration is increased in muscle treated with alcohol.

If a muscle is exposed for 8 to 9 minutes to 95 per cent alcohol, it shows little

change beyond the decrease of the water ring. Orientation is maintained in both the moist and the dried specimens, but the outside ellipse of the dried specimen is sharper than usual (Fig. 9).

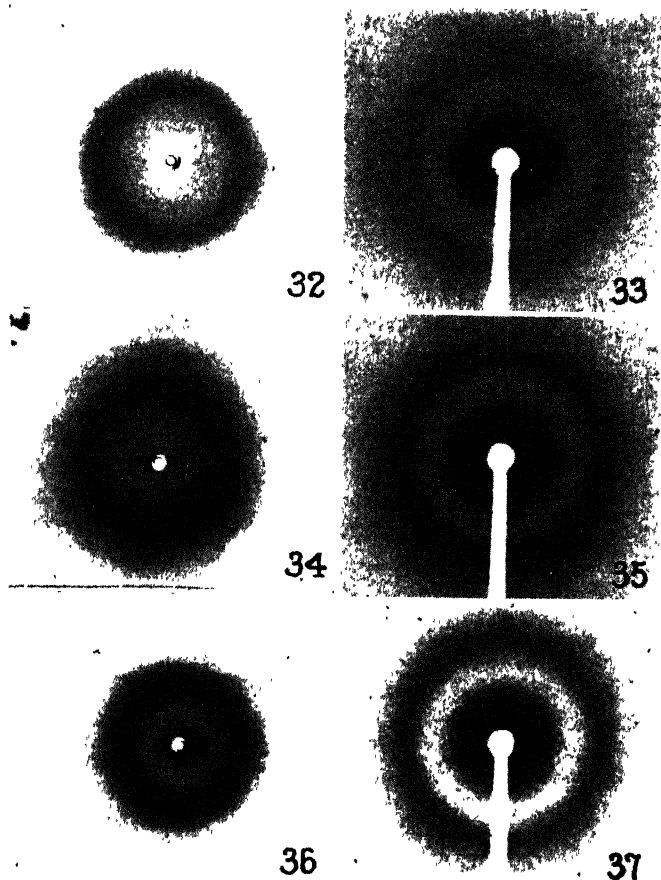


FIG. 9. Effects of alcohol. Nos. 32 and 33, muscle exposed 82 minutes to 5 per cent alcohol. Nos. 34 and 35, muscle exposed 44 minutes to 15 per cent alcohol. Nos. 36 and 37, muscle exposed 8 minutes to 95 per cent alcohol.

If the exposure time to 95 per cent alcohol is lengthened to 12 minutes, the sickles vanish although the streaks are still present. The elliptical character of the outside ring has entirely disappeared. It is as sharp as the corresponding ring in an x-ray diffraction pattern of boiled muscle. These findings are in good agreement with our observations on alcohol denaturation in proteins. These changes closely resemble qualitatively those produced by heat denaturation, although they seem quantitatively to be of lesser degree.



In order to study the effects of chloroform upon muscle different methods were used. Saturating the Ringer solution with chloroform by intensive shaking proved to be the most satisfactory way. The effect of chloroform upon the x-ray diffraction pattern of muscle depends upon whether the muscle is allowed to contract or not. Muscles tied in frames or weighted by 60 gm. do not show changes in the x-ray diffraction pattern after being kept overnight in Ringer's solution saturated with chloroform. The orientation of both the moist and the dried specimens is good. If, on the contrary, the muscle is allowed to contract in the chloroform-saturated Ringer's solution, which it does to a remarkable degree, then the diffraction pattern undergoes definite changes. The moist sample shows only faint streaks lying on a complete ring. The dried muscle shows, in its x-ray diffraction pattern, a ring instead of points. The elliptical character of the outside ring is lost. Instead of that, three diffraction rings corresponding to spacings of 21.2, 2.85, and 2.01 Å.u. become manifest. We have identified the last two spacings as belonging to NaCl and have described the first as occurring upon heat denaturation and other kinds of irreversible changes in muscle. These changes seem to be the effect of contraction. According to facts reported by Riesser (1925), the tension produced in muscle by chloroform is remarkably high, similar to the one reached in electrically induced tetanus. Muscles tied in frames and exposed to chloroform vapor for 10 minutes to 23 hours do not lose their orientation. Neither does chloralhydrate in 0.016 per cent solution in Ringer's solution affect the x-ray diffraction pattern of a muscle which was kept in such a mixture overnight.

A number of experiments were devoted to the study of the influence of caffeine upon the x-ray diffraction pattern of muscle. The caffeine concentration was varied from 0.002 to 0.01 per cent; muscles were exposed to these concentrations in Ringer's solution from 5 to 25 minutes. The muscles thus treated were either loose, pinned on cork, or allowed to contract isotonicly (12 exposures). The following results were found: Loose muscles when exposed to 0.002 caffeine did not show any changes of the x-ray diffraction pattern after an immersion time of 5 minutes. If this latter is extended to 25 minutes then the muscle shrinks to  $\frac{2}{3}$  of its original length. After drying the x-ray diffraction pattern shows a complete loss of orientation, the equatorial points are replaced by a ring; the outer ring is circular without any sickles. A number of salt rings are visible. Their spacings correspond to the ones of NaCl. We ascertained that they are different from the rings shown by powdered caffeine-sodium benzoate.

If the muscle is pinned on cork, the above used concentrations of caffeine and immersion time do not apparently influence the x-ray diffraction pattern. The orientation is as distinct as in the control. These fundamental facts are not changed either by increasing the concentration of the caffeine to 0.01 per cent, by tying the muscle in a frame, or letting it contract isotonicly. Only in one instance, when the moist muscle was tied in a frame and exposed to 0.01

per cent caffeine for 5 minutes did we notice a shrinkage of the water ring as upon acid or alcohol treatment. After drying, this same preparation showed a fine arc corresponding to a spacing of 4.18 Å.u. It seems to be an accepted fact that the caffeine prevents the restitution of lactacidogen in the muscle and therefore produces acid swelling. The changes described in the x-ray diffraction pattern of caffeine-treated muscles apparently confirm this hypothesis and explain why other agents which produce increase of acid by a different mechanism show identical x-ray diffraction patterns.

Although it is well known that acetylcholine acts only on the nerve terminals of muscle a few experiments (6) were made in this direction. Exposures to 0.001 per cent acetylcholine for 1 minute did not affect the x-ray diffraction patterns of either loose muscles or muscles tied in frames.

#### SUMMARY

1. X-ray diffraction studies of sartorius muscles of *Rana pipiens* were made in a new x-ray diffraction camera which permits exposures of 3 to 6 minutes. The object-film distance can be varied from 20 to 80 mm; the muscle inside the camera can be electrically stimulated while contracting isotonically or isometrically, and can be observed by a special device. After exposures up to 30 minutes (approximately 40,830 r) muscles are still alive and responsive.

2. Contrary to the x-ray diffraction pattern of powdered dry muscle, which pattern consists of two rings corresponding to spacings of 4.46 Å.u. and 9.66 Å.u., both moist and dried whole sartorius muscle show signs of orientation in both rings, consisting of two equatorial streaks (wet) or points (dry) and meridional sickles. The moist muscle shows in addition a diffuse water ring. The spacings corresponding to the orientation points and elliptical structure show only slight differences in moist and dried samples. Through statistical computations based on two different series consisting of thirteen moist and twenty-eight dried samples, and nine muscles before and after drying, it was shown that only the divergence in the smaller spacing has some real significance, which indicates that most water of the moist muscle is bound intermolecularly. Upon resoaking of dried muscle the x-ray diffraction pattern of the moist muscle is restored.

3. Stretching of muscle by weights below the breaking point produces an additional well defined diffraction line, corresponding to a spacing of 4.32 Å.u. A similar diffraction line can be produced in frog tendon upon stretching.

4. The influence of heat on the x-ray diffraction pattern of muscle depends upon the maximum temperature and the length of action; 5 minutes at 50° C. markedly reduces the orientation of the sample; 5 minutes' immersion in boiling Ringer's solution destroys the orientation and produces a ring corresponding to a spacing of 5.3 to 5.5 Å.u. in the moist and sharpening of the backbone reflection in the dried specimen.

5. Ultraviolet light brings forth changes in the x-ray diffraction pattern varying with the intensity of the irradiation. Ultimately a disappearance of the equatorial points and of the outside sickles is achieved while the elliptical shape of the outside ring and its diffuseness persist. In addition two salt rings characteristic of NaCl indicate that the irradiated muscles have become permeable to the surrounding medium (Ringer's solution).

6. Both faradic and single shock electrical stimulation were tried on muscles. If shortening of the muscle is prevented either by sufficient weight or by tying the muscle in a frame, no changes in the x-ray diffraction pattern occur; if the muscle is allowed to shorten without weights or by using insufficient weights, then the orientation either disappears completely or partially. When the muscle is stretched while contracted by electrical stimulation the orientation of the x-ray diffraction pattern reappears.

7. A number of salts with uni- and bivalent ions in concentrations corresponding osmotically to 0.73 per cent NaCl and 10 per cent  $\text{NH}_4\text{Cl}$  were studied in their effects upon the x-ray diffraction of muscles. Of the salts with univalent ions in the lower concentration only KCl causes a marked decrease of orientation and an increase in the permeability of the fiber membranes. Similar effects on the orientation seem to be produced by  $\text{CaCl}_2$  while  $\text{MgCl}_2$  causes rather a more pronounced orientation. At hypertonic salt concentrations the orientation disappears completely and the corresponding salt rings become visible. Besides, NaCNS seems to have a specific effect on the outside ring and LiCl produces a ring at 21.3 Å.u. and a splitting of the outside ring.

8. Strong mineral and lactic acids in concentrations up to 0.005 N have little if any influence upon the x-ray diffraction of muscles. A further increase in acidity to 0.01 N and above destroys the orientation completely, causes sharpening of the backbone reflection, and increased membrane permeability. These changes are irreversible upon neutralization. Also the effects of swelling upon the water ring of fresh muscle become manifest. Weak acids at higher concentrations show an effect similar to that of strong acids.

9. Rigor mortis produces a more or less complete loss of orientation. The muscles show signs of increased permeability.

10. Alkalies destroy the orientation of the x-ray diffraction pattern. The effective concentration is higher than the corresponding amount of acid.

11. Formaldehyde produces only minor changes in the x-ray diffraction patterns of muscles.

12. The effects of alcohol depend primarily upon the concentration applied. Low concentrations (5 per cent) seem to have a passing stimulating effect, at concentrations of 15 per cent, the anesthetizing effect becomes manifest in well defined orientation. The diameter of the water ring is reduced. If 95 per cent alcohol is allowed to act upon muscle for more than 12 minutes, then the orientation disappears completely and the backbone spacing becomes as sharp as in boiled muscle.

13. The effects of chloroform depend upon whether the muscle is allowed to contract or not. Only if the muscle is allowed to contract in chloroform-saturated Ringer's solution is the orientation lost and salt rings appear as well as a ring corresponding to a spacing of 22 Å.u., which has been observed in other changes in muscles.

14. In muscles allowed to shorten in a caffeine-Ringer's solution the orientation disappears, salt rings become visible as well as a decrease in size of the water ring; a new arc corresponding to a spacing of 4.18 Å.u. was observed in one case.

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# THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

## XII. THE PREPARATION AND PROPERTIES OF "MEGAPERMESELECTIVE" PROTAMINE COLLODION MEMBRANES COMBINING HIGH IONIC SELECTIVITY WITH HIGH PERMEABILITY

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### I

The preceding paper<sup>1</sup> of this series describes the preparation of "megapermselective" collodion membranes which are extremely impermeable to anions, but at the same time are very permeable to univalent cations. The purpose of the present paper is to describe the preparation and the properties of analogous electropositive "megapermselective" membranes which are very impermeable to cations, but at the same time very permeable to univalent anions.

Abrams and Sollner<sup>2</sup> have recently shown that electropositive membranes similar to the activated dried collodion membranes<sup>3</sup> can be prepared by the adsorption of protamine on highly porous collodion membranes and their subsequent drying in air. Such membranes show considerable electropositive activity in solutions ranging from pH 2-10. The "characteristic concentration potential" (0.1 M KCl/0.01 M KCl) of these membranes varied with different specimens from -47 to -52 mv.;<sup>4</sup> the membranes retained their electromotive properties on prolonged contact with water and electrolyte solutions. Their resistance was high and correspondingly their absolute permeability was low, having approximately the same magnitude as that of "activated" collodion membranes.<sup>3</sup> The dried protamine collodion membranes of Abrams and Sollner were of irregular shape and quite wrinkled in appearance; they were fragile and liable to easy destruction. It was also impossible with these membranes to measure accurately the surface area which should be known for many experiments. The present paper describes membranes which are free of these enumerated shortcomings. They are designated "permselective" or "megapermselective" protamine collodion membranes.

<sup>1</sup> Carr, C. W., and Sollner, K., *J. Gen. Physiol.*, 1944, **28**, 119.

<sup>2</sup> Abrams, I., and Sollner, K., *J. Gen. Physiol.*, 1943, **26**, 369.

<sup>3</sup> Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **25**, 7.

<sup>4</sup> To indicate that the concentration potential is opposite in sign to that of the negative membranes, the concentration potential values in the case of the positive membranes are preceded by a minus sign.

## II

In the present investigation we have combined the method used for the preparation of megapermselective collodion membranes described in the preceding paper<sup>1</sup> with the technique of Abrams and Sollner<sup>2</sup> for the preparation of positive membranes. With this new combined technique megapermselective protamine membranes are obtained consistently which combine great absolute permeability with significantly higher "characteristic concentration potentials" than those obtained in the earlier work;<sup>2</sup> these membranes also have a well defined shape, are smooth in appearance, and can stand considerable handling without danger of breakage.

Three-layer porous collodion membranes were cast on the outside of rotating tubes in exactly the same manner as described for the preparation of megapermselective collodion membranes.<sup>1</sup> A 4 per cent solution of Baker Collodion U.S.P. in ether-alcohol (50:50) was used. The porous membranes still on the tubes were immersed in repeatedly changed distilled water for 1 hour to remove the organic solvents. Then the membranes were placed in a 2 per cent solution of protamine sulfate<sup>5</sup> in a 0.025 M sodium borate-sodium hydroxide buffer of pH 11 and placed in an ice box at  $+2^{\circ}$  to  $+5^{\circ}\text{C}$ . After 48 hours they were taken from the solution, washed thoroughly, and dried for 5 hours in air while still on the tubes. Next, they were soaked in distilled water for several hours. Longer soaking in water than with the negative membranes was usually required before the membranes could be taken off the glass tubes. (Occasionally it was necessary to dip the membranes in 98 per cent alcohol for a few minutes to facilitate this removal.<sup>6</sup>) After this had been done, the membranes were fitted with glass rings for easier handling. Only a fraction of the membranes had at this stage the desired degree of ionic selectivity.<sup>7</sup> Further drying in air for at least 3 hours without support regularly results in membranes

<sup>5</sup> The authors are indebted to Eli Lilly and Company, Indianapolis, Indiana, for furnishing samples of this material.

<sup>6</sup> This brief immersion in 98 per cent alcohol swells the protamine covered membranes just enough to facilitate their easy removal from the tubes. Collodion membranes under similar treatment swell strongly and become quite soft and flabby. The absorbed protamine obviously greatly retards the alcohol in reaching the collodion. This interesting effect throws some light on the compactness of such adsorption layers. It warrants further investigation from the point of view of surface chemistry.

<sup>7</sup> The membranes in this state show great differences in selectivity. They give only in rare instances "characteristic concentration potentials" (0.1 M KCl/0.01 M KCl) of  $-52$  mv. or better, though their ionic selectivity is frequently satisfactory with 0.005 N and more dilute solutions. Their resistance is always very low. For work in electrolyte solutions of 0.005 N or lower normality they are therefore in many instances satisfactory, particularly when emphasis must be laid on the lowest possible resistance.

having fully satisfactory electrical and mechanical properties; though perceptibly shrunken they are perfectly smooth and test tube-shaped. These megapermselective protamine membranes are kept either dry or in water to which a crystal of thymol has been added as a preservative. The membranes frequently show a yellowish or brownish discoloration; this however does not affect their other properties or their usefulness. The megapermselective protamine membranes seem to be indefinitely stable when kept in air or water; they also show only very slow deterioration in electrolyte solutions. They can be considered as the electropositive analogues of the (electronegative) megapermselective collodion membranes described in the preceding paper.<sup>1</sup> At present the preparation of the megapermselective protamine membranes requires some skill and patience for optimum results. But there is little doubt that additional experience, as the membranes are used for further work, will gradually eliminate some of the still existing difficulties.

The limits within which changes in the described technique can be made are not nearly so wide as with the negative membranes. The conditions of preparation are apparently critical. The casting of the porous membranes is important. If they are too dense, only low concentration potentials are obtained, probably because many pores are too narrow to admit the protamine molecules. To obtain sufficient porosity with the brand of collodion used the ratio of alcohol to ether in the collodion solutions had to be equal to or somewhat greater than unity. The membranes after drying should have a thickness of 30 to 50  $\mu$ . Membranes thinner than 30  $\mu$  in many instances give low characteristic concentration potentials; they also break more easily on handling. Thicker membranes frequently give low concentration potentials presumably because the protamine had not been able to penetrate sufficiently. The new type positive membrane requires the use of protamine solutions of higher concentration than those recommended by Abrams and Sollner. With 0.2 to 0.5 per cent solutions only occasionally were membranes obtained giving characteristic concentration potentials of  $-52$  to  $-53$  mv. With 2 per cent solutions, however,  $-52$  to  $-53$  mv. was obtained consistently. For optimum results the pH of the protamine solutions should be controlled. In unbuffered solutions, the pH may change by two units, due to the reaction of the alkali solution with the collodion. The use of buffers is therefore indicated. The optimum pH range is rather broad. Above 11.5 the action of the alkali is too severe resulting in very weak membranes; below 10.5 the adsorption becomes too weak to give membranes with the highest selectivity. Membranes prepared in protamine solutions of pH 10.5–11.5 are satisfactory. The buffered protamine solutions can be used repeatedly because only a small amount of the protamine is adsorbed. After repeated use their pH should be adjusted if necessary. The protamine solutions should be kept at ice box temperature, whether in use or not.

In Table I are given the characteristic concentration potentials of some representative megapermselective protamine membranes prepared according to the above given procedure, as well as their resistances when immersed in 0.1 M KCl. The resistances are given as ohms per membrane of about 50 cm.<sup>2</sup>



active area. The potential and resistance measurements were carried out in the manner indicated in the preceding paper.<sup>1</sup> The resistance of these membranes assumes its final, lowest value only slowly, the figures in Table I representing approximately equilibrium values. This time effect warrants further investigation.

TABLE I  
*Characteristic Concentration Potential and Resistance of "Megapermselective" Protamine Colloid Membranes*

Membrane	Characteristic concentration potential 0.1 M KCl/0.01 M KCl $\pm 0.1$ mv.	Resistance in 0.1 M KCl solution $\pm 0.5 \Omega$
	<i>mv.</i>	$\Omega/50 \text{ cm.}^2$
a	-52.9	10
b	-53.0	12
c	-52.5	14
d	-53.0	12
e	-52.8	10
f	-53.0	5
g	-53.0	2 $\pm 0.2$
h	-52.8	0.5 $\pm 0.2$
i	-53.0	0.5 $\pm 0.2$

### III

Table I shows that the megapermselective protamine membranes consistently combine desirable electromotive properties with satisfactorily low resistance. It is three to four orders of magnitude lower than the resistance of the dyestuff- or alkaloid-impregnated positive membranes described in the literature. The "characteristic concentration potential" reaches in all cases -52 to -53 mv., the resistance in 0.1 M KCl solution being 0.5 to 15  $\Omega$  per 50 cm.<sup>2</sup> of membrane.

Shape, strength, and stability of these membranes as was indicated above are fully satisfactory.

It must be noted that the characteristic concentration potential of these membranes never reaches the thermodynamically possible maximum of -55.1 mv., the "cation leak" corresponding to -52 and -53 mv. being about 3 and 2 per cent respectively.<sup>1</sup> We shall take up this problem in the next section.

The new megapermselective protamine membranes were also tested for their absolute permeability in a few preliminary experiments on anion exchange across them. A membrane, showing a characteristic concentration potential of -53 mv. and a resistance of 2  $\Omega$  in 0.1 M KCl was filled with 30 ml. of 0.1 M  $\text{NH}_4\text{Cl}$  solution and placed in 30 ml. of 0.1 M  $\text{KNO}_3$ . After 4 hours 0.8 milliequivalent of  $\text{Cl}^-$  had exchanged through the membrane, whereas only 0.025 m.eq. of  $\text{NH}_4^+$  had entered the  $\text{KNO}_3$  solution. This "cation leak" corresponds to

3.2 per cent. Contrary to the situation found with the megapermselective collodion membranes, the positive membranes also show an appreciable leak of univalent cations in more dilute solution. The leak of bivalent cations as  $\text{Ca}^{++}$  or  $\text{Ba}^{++}$  is always much smaller than of the univalent cations.

## IV

The highest characteristic concentration potential that has been obtained with the megapermselective protamine membranes is  $-53$  mv. Since this value was obtained rather consistently, some further experiments were carried out to gain if possible some insight into the causes of this peculiarity of the protamine membranes.

TABLE II  
*Concentration Potentials at Different KCl Concentrations across "Megapermselective" Collodion and Protamine Collodion Membranes*

Concentration of KCl solutions		Concentration potential		
$C_1$	$C_2$	Calculated maximum values	"Megapermselective" collodion membrane $\pm 0.1$ mv.	"Megapermselective" protamine collodion membrane $\pm 0.1$ mv.
<i>mols/l.</i>	<i>mols/l.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>
0.64	0.32	15.9	12.6	-11.5
0.32	0.16	16.0	14.4	-13.8
0.16	0.08	16.2	15.6	-14.3
0.08	0.04	16.3	16.3	-15.8
0.04	0.02	16.6	16.6	-16.0
0.02	0.01	16.9	16.9	-16.2
0.01	0.005	17.1	17.1	-16.2

It is well known that the ionic selectivity of membranes increases rapidly as the absolute concentration of the electrolyte is decreased. Collodion membranes which show relatively low ionic selectivity in 1 M solutions become nearly completely anion-impermeable in 0.01 or 0.005 M solutions. To study this effect with the positive megapermselective membranes a series of concentration potential measurements was made with solutions having a 1:2 concentration ratio. To be able to compare the results with the behavior of negative membranes under similar conditions, parallel measurements were made with megapermselective collodion membranes. The results are given in Table II. The thermodynamically possible maximum values of the concentration potentials at the different concentration levels are also included in Table II. The activity coefficients used for the calculation of these values were taken from standard tables.

Table II indicates that the maximum thermodynamically possible value cannot be reached with protamine membranes even at very low concentrations.

The megapermselective protamine membranes show a significant cation leak<sup>1</sup> under all conditions.

The obvious and simplest explanation is found in the assumption that some of the critical spots in the heterogeneous<sup>8</sup> pores of the membrane do not carry a positive charge. They may be either uncharged or carry a negative charge. This could be due to several different factors. The protamine molecules having a molecular weight of around 3000 are probably too large to enter many narrow constrictions of the pores even of the porous membranes; thus some critical spots would not carry any positive charge. The alkali and  $\text{OH}^-$  ions which are present in the same solution, however, can reach many narrow places and oxidize the pore walls there and thus leave them negatively charged. Or, the protamine carries inherently, as is most likely the case, some carboxyl groups which would occupy the critical spots in some pores; or, the protamine contains some impurity carrying acidic groups which would bring about the same result. At present no decision can be made between these different possibilities. We plan to study this problem further, mainly by the use of different protamine preparations.

V

The availability of the electropositive megapermselective protamine membrane opens up a still wider field for further investigation than is opened up by the availability of the electronegative megapermselective collodion membrane, for much less is known about the properties of electropositive than of electronegative membranes.

The problems which can successfully be attacked now are quite analogous to those mentioned in connection with the megapermselective collodion membrane.<sup>1</sup> They do not need to be enumerated again.

However, it may be mentioned that megapermselective protamine collodion membranes have been used already with considerable success in the potentiometric determination of anions, such as  $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{I}^-$ ,  $\text{F}^-$ ,  $\text{ClO}_3^-$ ,  $\text{ClO}_4^-$ ,  $\text{BrO}_3^-$ ,  $\text{IO}_3^-$ ,  $\text{NO}_3^-$ , and  $\text{Ac}^-$ .<sup>9</sup> For the determination of several of these ions no other potentiometric method is known.

The availability of both positive and negative membranes of high ionic selectivity and great permeability now also permits the experimental investigation of various mosaic structures,<sup>10</sup> which may play an important rôle in biological processes. The theory of some mosaic systems was worked out several years ago;<sup>11</sup> for lack of suitable membranes, however, it could not be tested heretofore.

<sup>8</sup> Sollner, K., and Anderman, J., *J. Gen. Physiol.*, 1944, **27**, 433. Sollner, K., and Carr, C. W., *J. Gen. Physiol.*, 1944, **28**, 1.

<sup>9</sup> Sollner, K., *J. Am. Chem. Soc.*, 1943, **65**, 2260.

<sup>10</sup> Höber, R., and Hoffmann, F., *Arch. ges. Physiol.*, 1928, **220**, 558.

<sup>11</sup> Sollner, K., *Biochem. Z.*, Berlin, 1932, **244**, 370.

The successful preparation of the megapermselective protamine membranes suggests the possibility of the preparation of analogous membranes in which various proteins, peptones, and deaminated proteins are used in place of the protamine. Such protein membranes, like the protamine membranes, would contain embedded in a rigid framework substances of particular biological interest which under conditions somewhat analogous to those found in living organisms could thus be investigated conveniently for their electromotive behavior. The use of deaminized proteins should permit the study of strongly electronegative membranes, more comparable to natural membranes than collodion.

#### SUMMARY

The technique of Abrams and Sollner for the preparation of electropositive dried protamine collodion membranes has been improved. Porous collodion membranes cast on the outside of rotating tubes are treated for 48 hours with a solution of 2 per cent protamine sulfate buffered at pH 11. After being washed thoroughly the membranes are dried in air for several hours, soaked in water for several hours, and removed from the tubes. Further drying in air but without support shrinks the membranes slightly. The resulting membranes are designated "permselective" or "megapermselective" protamine collodion membranes. These membranes regularly give characteristic concentration potentials of  $-52$  to  $-53$  mv. and (in  $0.1\text{ M KCl}$ ) resistance of  $0.5$  to  $15$  ohms per membrane of  $50\text{ cm.}^2$  area. This resistance is several orders of magnitude smaller than that of the conventional dyestuff- and alkaloid-impregnated positive membranes. The megapermselective protamine collodion membranes can be kept either dry or in water for prolonged periods without detectable deterioration. They are quite smooth, have a regular shape, and stand considerable handling without breakage.

The megapermselective protamine collodion membranes are the electropositive analogues of the electronegative megapermselective collodion membranes described by Carr and Sollner.



## ELECTRIC TISSUE

### RELATIONS BETWEEN THE STRUCTURE, ELECTRICAL CHARACTERISTICS, AND CHEMICAL PROCESSES OF ELECTRIC TISSUE

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#### INTRODUCTION

Several recent papers have shown comparisons between the results of electrical measurements made on the discharge of the electric eel, *Electrophorus electricus* (Linnaeus) and those of chemical measurements on the electric tissue (1-4). The present paper is concerned with the same research but will deal mainly with an analysis of the electrical measurements themselves and the relation between the electrical characteristics and the structure of the organs.

The electric eel offers several advantages in such an inquiry over the other electric species. In contrast to most of them, it can be kept in captivity. Its electrical characteristics are rather constant and do not change materially even when it is kept for some minutes out of water. Also its electric organs, being long and lying just under the skin, allow electric connections to be made at any points along their length, whereas the electric organs of the rays, *Torpedo* and *Narcine*, are conveniently accessible only at their poles. Thus, in the organs of the electric eel, it is possible to compare the electrical activity of their different parts. The observed electrical variations may then be studied in relation to the variations found in the dimensions of the organs and the arrangement of the electroplaxes.

#### *Organs of the Electric Eel*

The electric eel has three pairs of electric organs, the main organs, which extend along the posterior four-fifths of the length of the fish, the much smaller organs of Hunter, which lie under the main organs along their entire length, and the organs or "bundles" of Sachs, which lie above the main organs in the posterior half of the fish. The discharges of the main organs and of the organs of Sachs can be clearly distinguished from each other. Hunter's organs, on the other hand, seem to discharge with the main organs, which they closely resemble in the arrangement of the electroplaxes and from which they are only separated by a thin layer of muscle. In the present paper, wherever

the dimensions of the organs are involved, the organs of Hunter will be considered as a part of the main organs, and where the main organs are mentioned it is to be understood that Hunter's organs are included. The point is of no great importance, however, for the cross-section of Hunter's organs is so small that their exclusion would hardly change any of the significant results by more than the probable error.

#### DIMENSIONS OF THE SINGLE ELECTROPLAX LAYER

The variation in the dimensions of the organs is illustrated in Fig. 1, in which, for one specimen, their cross-sectional area is plotted as ordinate against the distance along the organs from anterior to posterior.

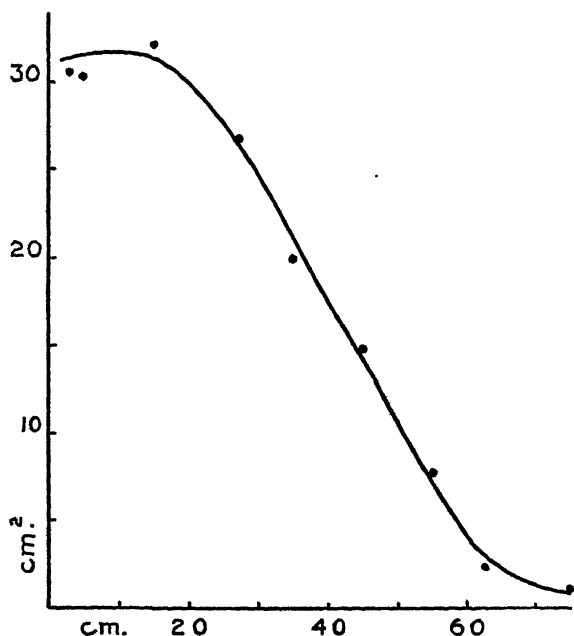


FIG. 1. Variation in cross-section of main organs. Area of cross-section (cm.<sup>2</sup>) vs. distance from anterior end (cm.).

The electroplaxes are arranged in a regular structure which has often been described. Plates showing sections of the organs at several places were included in one of the papers already cited (2). At any one cross-section the electroplaxes have a uniform thickness in the direction of the length of the fish, which is also the direction of the electric current in the discharge. But along the organs this thickness varies, the electroplaxes at the anterior end being very much thinner than those at the posterior end. In Fig. 2a the number of electroplaxes per unit length along the organs is plotted, as the

cross-sectional area was plotted in Fig. 1, against the distance from the anterior end. The likeness of the two figures is apparent. This likeness reflects a structural characteristic which is most simply described by considering a

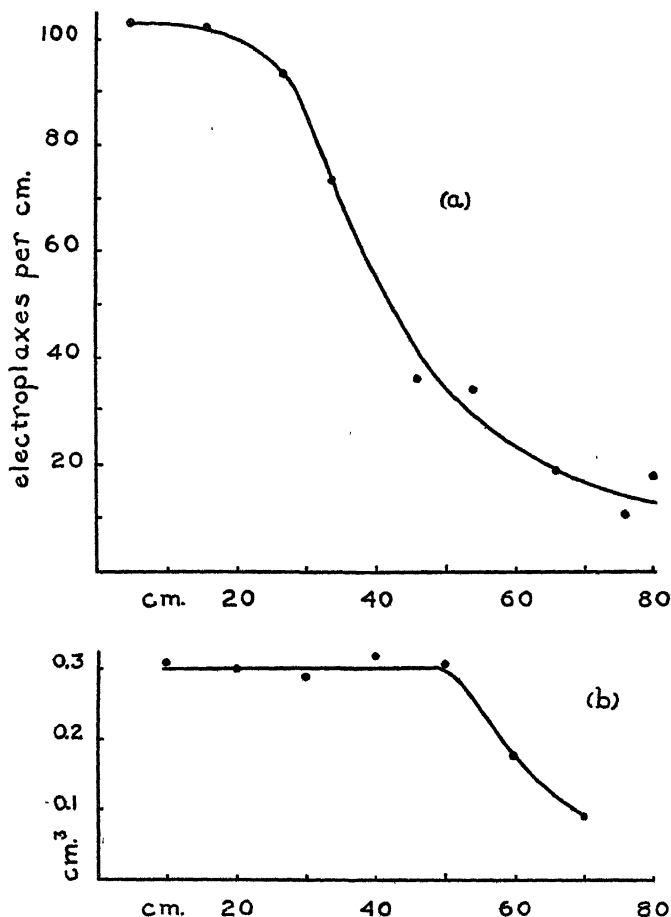


FIG. 2. Variation in packing of electroplaxes in main organs. (a) Number of electroplax layers per cm. (b) Volume of single electroplax layer (cm.<sup>3</sup>), vs. distance from anterior end (cm.).

slice across the organs one electroplax thick. From Fig. 1 such a slice will have an area decreasing from anterior to posterior, and from Fig. 2 it will have a thickness increasing in the same sense. The similarity of the two figures shows that the volume of the slice is roughly uniform along the organs, the decrease in area toward the tail being offset by the increase in thickness.



This volume, at any point along the organs, is found by dividing the cross-sectional area by the number of electroplaxes per unit length. The plotted points of Fig. 2*b* show volumes found in this way from readings on the smooth curves of Figs. 1 and 2*a*. Over most of the length of the organs the deviations from a uniform volume are no greater than the errors of the measurements. The break in the curve appears at a place on the organs at which their cross-section has diminished from the anterior end until it is comparable with that of the organs of Sachs at the same place. At points a little anterior to this place, the organs of Sachs are of much smaller cross-section than the main organs.

The uniformity in the volume of the single electroplax layer has been observed before (5). The purpose in presenting it again is to introduce a discussion of its relation to the electrical characteristics of the tissue somewhat more detailed and based on the observation of more specimens than that given before.

#### ELECTRICAL CHARACTERISTICS AT THE PEAK OF THE DISCHARGE

##### *Measurements with Open External Circuit*

The simplest of the electrical measurements is that of the peak voltage developed during the discharge between electrodes placed against the skin which covers the organs. A cathode ray oscillograph is used for the measurement. All measurements are made with the fish out of water. As might be expected, the peak voltage depends on the circuit external to the fish and has its greatest value when this circuit is open so that there is no external current. When the "maximum voltage" is mentioned in this paper, the peak voltage with the external circuit open will always be meant. If having the external circuit open prevented any current in the electric organs, then the maximum voltage would be the true open circuit voltage and would therefore be equal to the E.M.F. of the segment of the organs between the electrodes. But, whether the external circuit is open or not, closed circuits must exist during the discharge in the body of the fish. It is certainly to be expected that such circuits will be formed by the electric organs and the adjacent non-electric tissue together. Possibly also there may be closed circuits lying wholly within the electric organs. The current flowing in such circuits will be called the "leakage" current. Its effect is to cause a voltage drop in the organs, so that the maximum voltage will be somewhat less than the E.M.F. Although the leakage current is not directly measurable, there will be described in a later part of this paper a method of making allowance for its effect.

In the discharge of a long segment of the organs, the voltage will begin to decline at the anterior end before it reaches its peak at the posterior end. Thus the maximum voltage developed in such a segment is a little less than the sum of the maximum voltages developed in its parts. But the time lag

in a length of 5 or 10 cm. is too small to have an appreciable effect on the maximum voltage. For the voltage stays near its maximum for a time of the order of 1 msec., and the speed at which the impulse runs along the organs is of the order of 1 m. per msec., being somewhat greater at the anterior end and less at the posterior end (6). The voltage is thus near its peak simultaneously over a length of the order of 1 m. in a fish with organs of that length or more. Therefore, in a segment 5 or 10 cm. long, the maximum voltage developed between its ends may be divided by its length to give the maximum voltage per unit length.

In the observations next to be described, the length of the segment was taken as the distance between the nearer edges of the electrodes, 9 cm. with the larger fish and 5 cm. with the smaller. Fig. 3*a* shows the maximum voltage per unit length plotted against the distance from the anterior end of the organs, with the same fish as the one on which the measurements of Figs. 1 and 2*a* were made. The likeness of the graph of Fig. 3*a* to those of the other two figures is evident.

The maximum voltage per electroplax layer is found by dividing the maximum voltage per centimeter by the number of electroplax layers per centimeter. The values shown by the plotted points of Fig. 3*b* were found in this way from readings made on the curves of Figs. 2*a* and 3*a*. The maximum voltage per electroplax layer decreases from anterior to posterior but has everywhere the order of magnitude of 0.1 volt, a value familiar from studies of biological boundary potentials.

The fish thus far described was 103 cm. in length. Lately, in the course of a comparison of maximum voltage per unit length with concentration of choline esterase, we have made similar measurements on other electric eels (2). With three of these (Nos. 1, 2, and 4 of the paper just cited) the lengths of which were 51, 57, and 114 cm. respectively, both the cross-sectional area and the maximum voltage per unit length were determined at a number of points along the organs. The results of these measurements are shown in Fig. 4, those from fish 1 at the upper right, No. 2 at the upper left, and No. 4 below. Although these fish were so different in size, the proportionality between the cross-sectional area and the maximum voltage per unit length was found with all of them.

The number of electroplax layers per unit length was not determined in these three fish at enough points to provide graphs similar to those of Figs. 2 and 3*b*. For the points at which this measurement was made, the volume of the transverse layer one electroplax thick and the maximum voltage per electroplax layer were computed. The volumes of the single electroplax layer, in cubic centimeters, are shown in parentheses above the points on the graphs to which they correspond. The maximum voltages per electroplax layer are shown in brackets below. The volumes are naturally different for

the different fish but do not vary widely in any one. The maximum voltages per electroplax layer lie within extreme values 0.16 and 0.11 volt for all three fish.

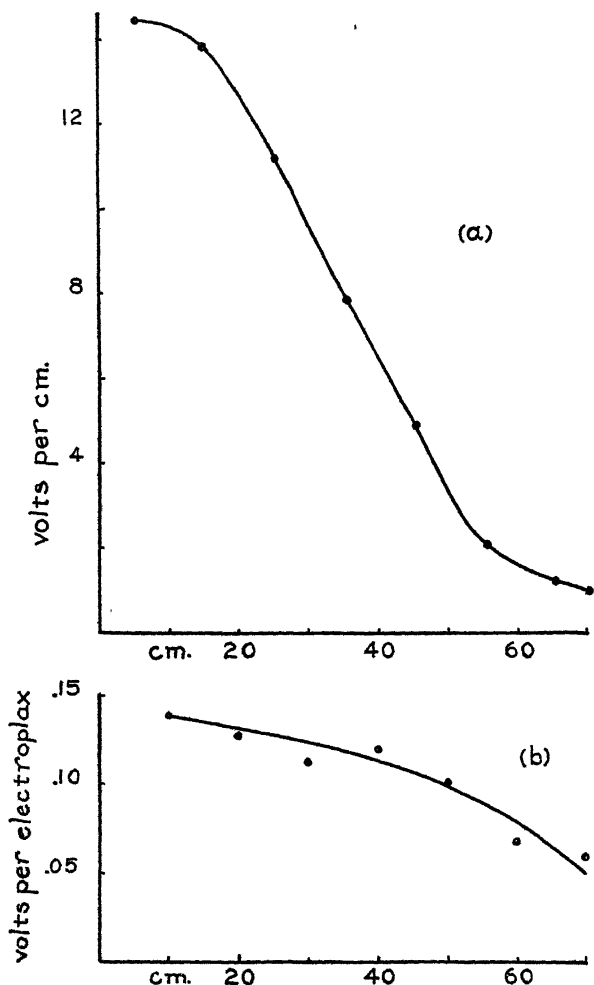


FIG. 3. Maximum voltages in main organs. Maximum voltage (a) per cm., (b) per electroplax layer vs. distance from anterior end (cm.).

#### *Measurement with Closed External Circuit*

Current may be drawn from the fish by joining a resistance between the electrodes. The peak voltage may be measured with the oscillograph as before. The resistance being known, the peak current may be calculated by Ohm's law,  $V = IR$ . When different values of the external resistance  $R$  are

used, so that the peak voltage  $V$  and the peak current  $I$  are both varied, it is found that the graph of  $V$  against  $I$ , as they are measured when care is taken not to tire the fish, approximates a straight line with both the electric eel

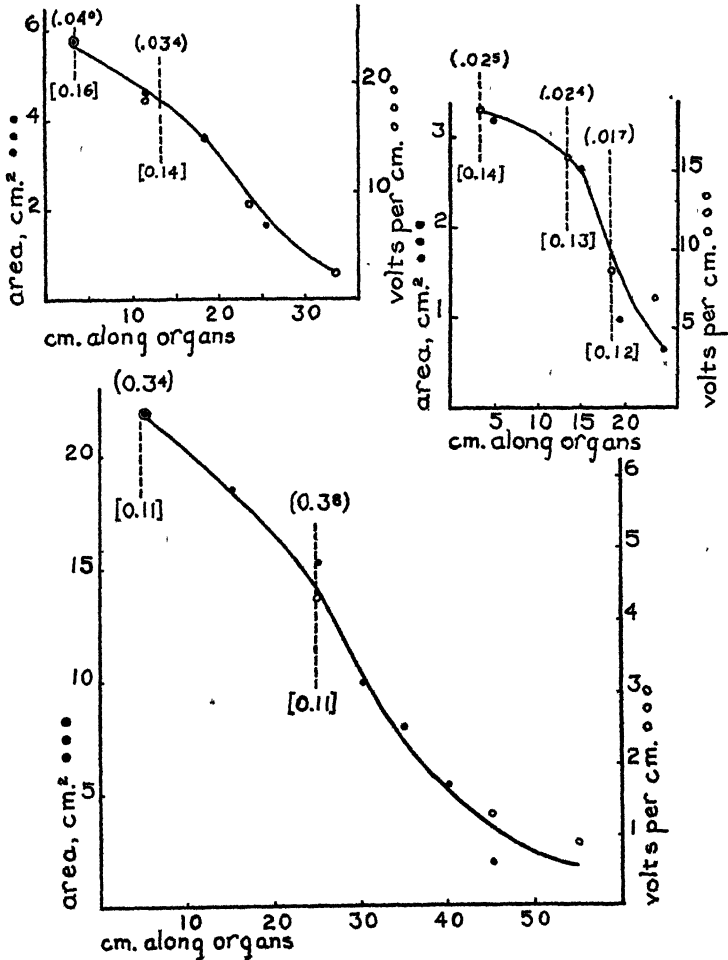


FIG. 4. Dimensions and maximum voltages in three specimens. See text for explanation.

and electric rays (2, 7, 8). This was to be expected if the organs can be described electrically in terms of E.M.F. and ohmic internal resistance. For let  $E$  be the E.M.F. and  $r$  the internal resistance of the segment of the organs between the electrodes. For the present we neglect the leakage current and assume that the current in the organs is equal to the current  $I$  in the external

resistance. On this assumption, the potential difference between the electrodes will be given by  $V = E - Ir$ , a linear relation between  $V$  and  $I$  if  $E$  and  $r$  are constants of the equation.

If, instead of neglecting the leakage current, we should assume only that the aggregate of electric and non-electric tissue can be described electrically as some network of E.M.F.'s and ohmic resistances, a linear relationship between  $V$  and  $I$  would still be expected. For by Thévenin's theorem of electric networks, any ohmic network will supply current to a resistance joining any two of its points as if it were a single E.M.F. and internal resistance. The equivalent E.M.F. will be equal to the voltage existing between the two points when no current is drawn from the network, and the equivalent resistance will be equal to the resistance of the network between the two points.

The fact that the measured values of peak voltage and peak current show a linear relationship may then be taken as evidence that the organs can be described electrically, at least at the peak of the discharge, as some arrangement of E.M.F.'s and ohmic resistances. It should not be assumed, however, that all the characteristics of the tissue will be shown by a study of its discharge. That the resistance is not ohmic under all conditions is shown by the fact that it acts as a rectifier to an external voltage opposed to and greater than its own (9). The boundary of nerve axon is also rectifying, as was shown by Cole and Baker (10). It is reasonable to expect, as has been generally assumed, that most characteristics of electric and nervous tissue will be similar. Thus, for example, it is likely that under the action of an alternating voltage of high enough frequency electric tissue will show an effective reactance as well as a resistance. For the present we are concerned with finding the electrical characteristics needed to describe the discharge, and for this purpose E.M.F. and ohmic resistance appear to suffice. But the additional assumption that the leakage current is negligible can be taken only as a provisional and crude approximation.

Making this assumption and using the equation  $V = E - Ir$ , we may use the measured values of  $V$  and  $I$  to find the value of  $r$ , the internal resistance of the segment of the organs between the electrodes. If  $V$  is plotted against  $I$  as in Fig. 5,  $r$  will be the downward slope of the graph. By changing the positions of the electrodes, the resistance of different segments of the organs can be determined. The method of making electric connection with the organs does not seem to be critical in the determination of the internal resistance, as the area of the electrodes in contact with the skin can be varied considerably without materially changing the measured peak voltage. This indicates also that the resistance of the skin is not an important part of the resistance  $r$ , for the resistance of the skin would vary inversely with its area in contact with the electrodes.

The resistance of a conductor of length  $L$  and cross-sectional area  $A$  is

given by  $r = \rho L/A$ , where  $\rho$  is the resistivity of the material of which the conductor is made. In the usual units it is the resistance in ohms between opposite faces of a cube 1 cm. on a side. The resistivity of the electric tissue may be estimated from the values of  $r$  obtained from the  $V - I$  graph by taking  $L$  as the distance between the electrodes and measuring the cross-sectional area of the organs. If the resistivity of the electric tissue were uniform, the values of  $r$  for segments of equal length would be expected to increase from anterior to posterior as the cross-section of the organs decreases. Actually, however, the resistance does not show any variation comparable with that of the cross-sectional area. The resistivity must therefore decrease in about

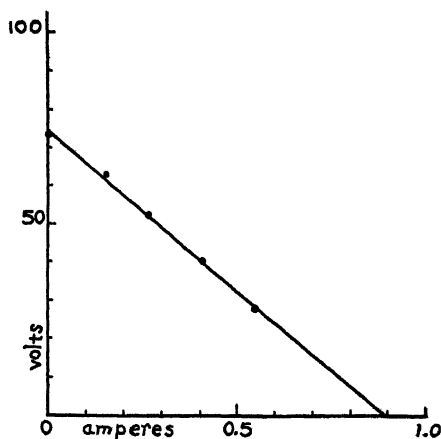


FIG. 5. Evidence of ohmic characteristics of tissue. External peak voltage vs. external peak current with various external resistances joining electrodes on main organs.

the same proportion as the area. It has been mentioned before that the number of electroplaxes per centimeter decreases in about this proportion. Fig. 6 shows the resistivity of the electric tissue of the fish of Figs. 1, 2, and 3, plotted against the number of electroplax layers per centimeter.

It will be seen that the plotted points lie near a straight line through the origin, so that the resistance of unit cube of electric tissue is proportional to the number of electroplax layers of unit area which it contains. The resistance of 1 cm.<sup>2</sup> of a single electroplax layer has thus a uniform value (about 5 ohms for this fish) whatever the thickness of the electroplax. This is an interesting result since much the most probable explanation for it is that the resistance of the electric tissue, at least at the peak of the discharge, is mainly in the boundaries of the electroplax. In Table I are given values of the resistance of 1 cm.<sup>2</sup> of single electroplax layer for the other electric eels already mentioned, together with an estimate for the electric ray, *Narcine brasiliensis*,

made from data of Cox and Breder and the lowest value reached during activity by the boundary of the squid giant axon, as given by Cole and Curtis (8, 11). However, only the order of magnitude of the measurements on the electric tissue is significant. The uncertainty resulting from the neglect of the leakage current has already been mentioned. In addition to this, there is the fact that the electroplax boundaries are highly convoluted and may have an area of much more than  $1 \text{ cm.}^2$  in  $1 \text{ cm.}^2$  of single electroplax layer. For

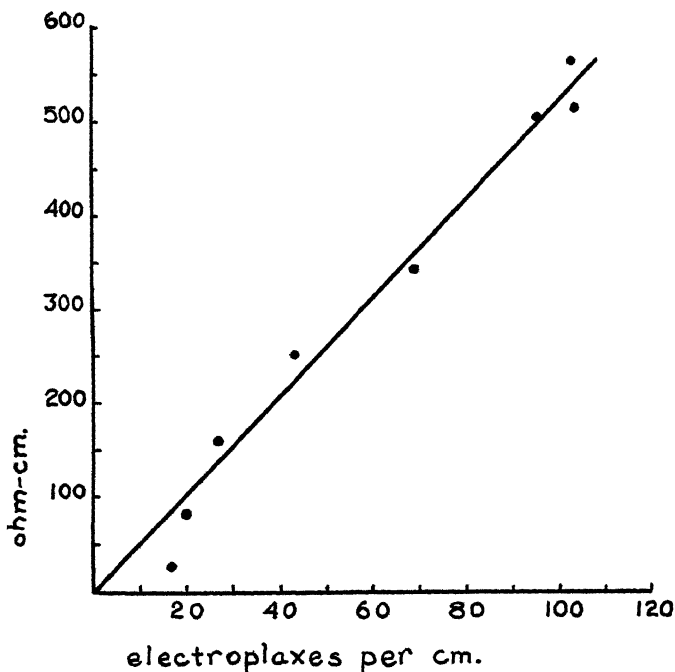


FIG. 6. Uniformity in resistance of unit area of electroplax layer. Resistivity of electric tissue (ohm-cm.) vs. number of electroplax layers per cm.

both of these reasons, the true value of the resistance of  $1 \text{ cm.}^2$  of electroplax boundary may well be several times greater than the values given above.

On the  $V-I$  graph of Fig. 5, the maximum current is given by the point at which the graph meets the horizontal axis. This is the current produced at the peak of the discharge when the segment of the organs is completely short-circuited by a negligible resistance joined between the electrodes. This current may be divided by the cross-sectional area of the organs in the segment between the electrodes to give the maximum current per unit area of cross-section. It is found that the maximum current per unit area is roughly uniform along the organs of any one fish. This follows from the rough uni-

formity, already mentioned, of the maximum voltage per electroplax and the resistance of unit area of the single electroplax layer. In the fish of Fig. 5 the maximum current per unit area had the average value 0.05 amp. per cm.<sup>2</sup>, and for fish 1, 2, and 4 the average values were respectively 0.04, 0.06, and 0.02 amp. per cm.<sup>2</sup> Values of 0.08 and 0.10 amp. per cm.<sup>2</sup> were found for two specimens of *Narcine*. An estimate of 0.24 amp. per cm.<sup>2</sup> has been made for a single specimen of *Torpedo occidentalis* (7). (In the papers from which the figures for *Narcine* and *Torpedo* were obtained, the values of current per unit area given were those for maximum external power. Since maximum external power is attained at half the maximum current, the values given above are twice those given in the papers.)

In the preceding calculations it has been supposed that the right and left organs discharge simultaneously. In *Narcine* and *Torpedo*, where the organs are far apart in the body of the fish, this has been shown to be true. In the

TABLE I  
*Values of the Resistance of a Layer 1 Cm.<sup>2</sup> in Area*

Species.....	<i>Electrophorus electricus</i>								<i>Narcine</i> <i>b</i> average adult	Squid, giant axon
Specimen No.....	1			2			4			
Distance along organs, cm.....	3.5	13.5	18.5	3.5	13.5	5	25			
Resistance of 1 cm. <sup>2</sup> , ohms.....	3.2	3.6	2.3	3.4	2.2	4.7	4.5	1.1	25	

eel, the right and left organs are too near together for the use of the test made on the rays. Nevertheless it seems to us probable that the right and left organs of the eel discharge together, because, with the electrodes adjacent to the organs on one side, the oscillographic traces of successive discharges show no difference of a kind to suggest that some of them occur in the nearer and some in the more remote organ. If, contrary to what we suppose, it should be the fact that the right and left organs of the eel discharge separately, then the values given for the resistivity of the tissue and the resistance of unit area of the single electroplax layer should each be halved, and those given for the maximum current per unit area should each be doubled.

#### ELECTRICAL CHANGES DURING DISCHARGE

##### *Schematic Representation of Electric Tissue*

The electrical characteristics thus far discussed are only those at the peak of the discharge. It has been pointed out that these characteristics are related in more than one respect to the dimensions of the electroplax layer. This fact makes it seem reasonable to expect that the electrical changes taking



place during the discharge in each electroplax layer may be at least partly inferred from external electrical measurements on a segment of the intact organ. But before considering how the electrical characteristics of the tissue change during the discharge, it will be desirable to develop a method of allowing in the calculations for the effects of the leakage current.

The actual paths of this current must form an extremely complex network. Every part of the organs, during the discharge, is electrically positive with respect to every posterior part and electrically negative with respect to every anterior part. Also the organs are in electric connection along their whole length with the adjacent non-electric tissue. Hence we should expect, even

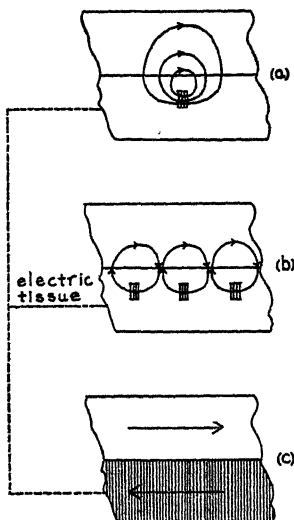


FIG. 7. Schematic representation of the internal currents.

without considering the possibility of closed circuits wholly inside the electric organs, that each small part of the organs, considered apart from the rest, would produce a current not confined to any single path or to any few paths. This is shown schematically in Fig. 7*a*, in which the shaded area represents the part of the electric tissue producing the currents shown.

Considering a number of such small regions in the organs, we should expect a condition shown schematically in Fig. 7*b*, where for simplicity only one path of the current has been shown through each small region considered. It is evident here that the currents into and out of the organs will partially cancel one another. If then we consider a continuous segment of the organs, not too near either end and short enough that the electrical characteristics of the tissue can be considered uniform along the length of the segment, it will be a fair assumption to consider the currents laterally into and out of the segment as cancelling one another, leaving only longitudinal currents, one way inside

the electric tissue and the other way outside, in the non-electric tissue, as shown in Fig. 7c.

Each of these currents will be uniform along the segment, and it will therefore be permissible to represent each as following a single path, without transverse connections except at the ends of the segment. It does not appear that there would be any necessary difference in this conclusion if circuits closed wholly within the electric tissue were considered also.

An external resistance connected between the electrodes will provide another path for the current. The presence of this connection may be expected to produce some distortion of the leakage current near the ends of the segment and thus impair the validity of the assumption just made that this current is uniform. But if the external resistance is high, this distortion will probably be slight, and if it is low the external current will be so large in comparison

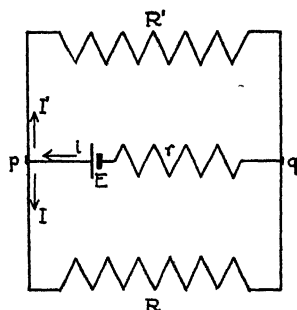


FIG. 8. Proposed equivalent circuit of electric tissue, leakage path, and external resistance. For meaning of symbols see text.

with the leakage current that a moderate error in the assumption made concerning the leakage current will probably not be serious. Consequently in either case we may hope to represent the actual conditions fairly well by the circuit shown in Fig. 8, in which  $E$  denotes the E.M.F. of the electroplax in series between electrodes at  $p$  and  $q$ ,  $r$  denotes the internal resistance of the electric tissue,  $R'$  the resistance of the leakage path, and  $R$  the external resistance. Since the discharge is intermittent, it is clear that at least one, if not both, of the quantities  $E$  and  $r$ , which characterize the electric tissue, must be variable.

At any one instant during the discharge, let the currents in the resistances  $r$ ,  $R'$ , and  $R$  be denoted by  $i$ ,  $I'$ , and  $I$  respectively. Of these three currents only  $I$ , the current in the external resistance, is found directly from the measurements. It is known from the relation  $V = IR$ , where  $V$  is the voltage between the electrodes, as measured with the oscillograph. The voltage is related to the currents in the other two branches by the two equations

$$V = I'R' \quad V = E - ir$$

Since  $i = I + I'$ , the second of these equations may be written  $V = E - Ir - I'r$ . If  $I'$  is replaced by  $V/R'$ , its value according to the first equation, we obtain  $V = E - Ir - Vr/R'$ , which may be written  $V(1 + r/R') = E - Ir$  or, dividing by  $r$ ,  $V(1/r + 1/R') = E/r - I$ . It is convenient now to let  $1/r + 1/R' = 1/r'$ . The new resistance  $r'$  is simply the resistance of  $r$  and  $R'$  in parallel. Making this substitution and multiplying by  $r'$ , we obtain

$$V = Er'/r - I'r'$$

This is a linear relation between  $V$  and  $I$ , provided the resistances are ohmic. To test it, the voltage and current at a given instant in one discharge must be compared with those at the corresponding instants in other discharges through different external resistances. The comparison is made by measurements on oscillographic traces like those shown in Fig. 9.

#### *Method of Measurement*

The comparison requires first of all that corresponding instants on the different traces be identified. If the instant at which the discharge starts could be clearly distinguished on the oscillographic traces, corresponding instants would be those at equal intervals after the start. It is evident from the traces shown that the instant at which the discharge starts cannot be precisely determined. But it will also be noticed that a very short while after the beginning there is an interval during which the voltage rises at a nearly uniform rate, as shown by the nearly straight rising branch of the oscillographic trace. Extending this straight portion back until it meets the base line determines a point which may be taken as marking the zero time from which intervals are to be measured.

This procedure is illustrated in Fig. 10, in which one trace from each row of Fig. 9 has been copied. The zero times of the three traces have been found in the manner just described, and the traces have been shifted until the three zeros coincide on the time scale. The ordinates of the traces at 1 msec. give three values of the voltage  $V$ , and the quotients of the voltages by the resistances give the corresponding values of the current  $I$ . Thus three points are determined on a graph of  $V$  against  $I$ , for a comparison with the linear equation  $V = Er'/r - I'r'$ .

Actually in obtaining the results now to be described, measurements made on a number of traces were averaged to determine each point. The traces were obtained in the following way. The fish was removed from the water and placed in contact with two electrodes 10 cm. apart, which were connected to the cathode ray oscillograph. While the fish was made to discharge by light prodding, different resistances were connected between the electrodes, and the screen of the oscillograph was photographed. Five or six exposures were made with the external circuit open and as many with each of the resistances 400, 200, and 100 ohms, and with the oscillograph at a higher gain five or six were made with each of the resistances 100 and 50 ohms. In order to avoid any effect of fatigue on the discharge, the number of discharges was kept within a limit which had been found safe in other observations, and the whole procedure was carried through in the shortest time possible, 2 or 3 minutes.

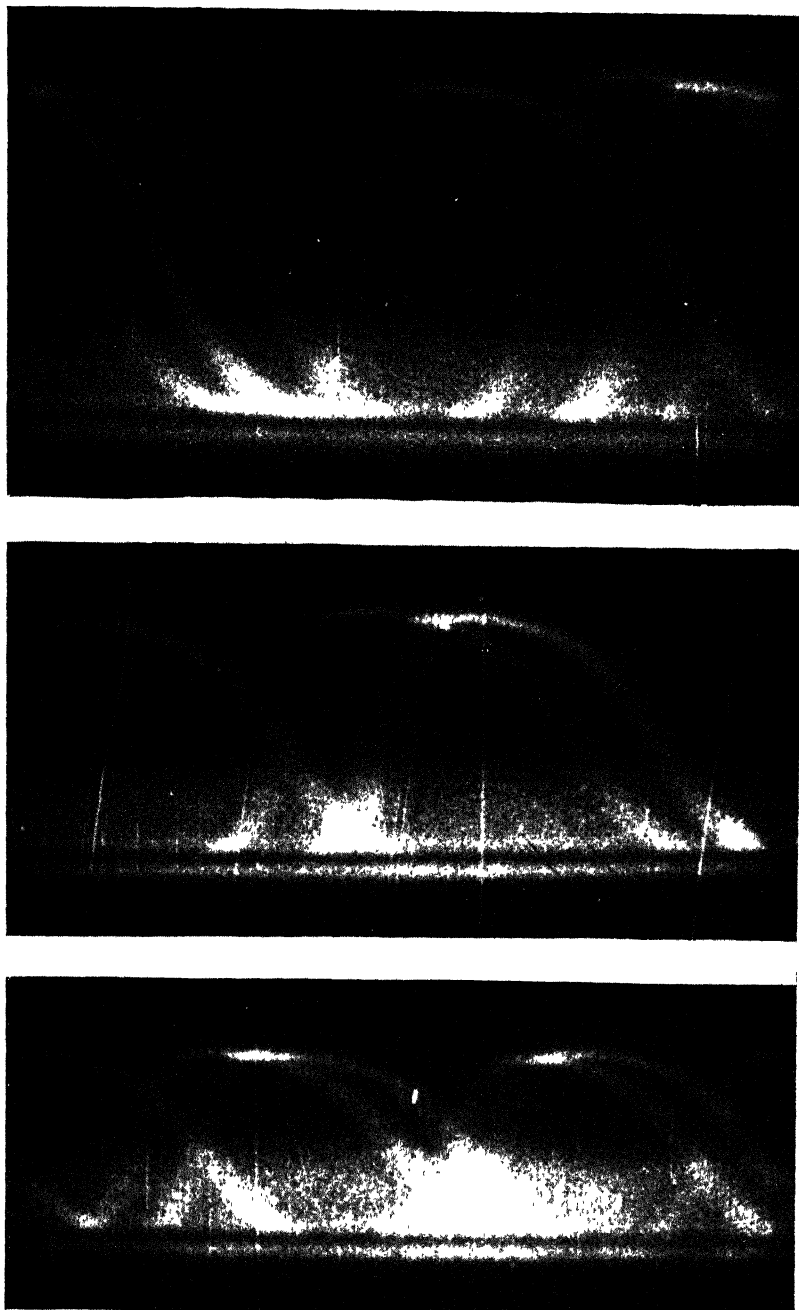


FIG. 9. Oscillograph records of discharge of segment of main organs. External circuit open (top), closed through 200 ohms (middle), through 100 ohms (bottom). For scales of voltage and time see Fig. 10.

To insure that any fatigue, if it did occur, would have no systematic effect on the results of the experiment, the order in which the different resistances were connected to the fish was reversed in alternate series of exposures.

Some of the traces were obscure because of overexposure or excessive overlapping and others were incomplete because the discharge occurred at the beginning or end of the timing sweep of the oscillograph. Of those that were complete and legible on the film a certain number, greater in some series of observations and less in others but about one in six on the average, were very different from the rest either in height or pulse form or more often in both. Most of the traces are quite uniform and the anomalous ones are usually distinguished without any trouble. Their difference is so marked that it once appeared reasonable to suppose that they showed the dis-

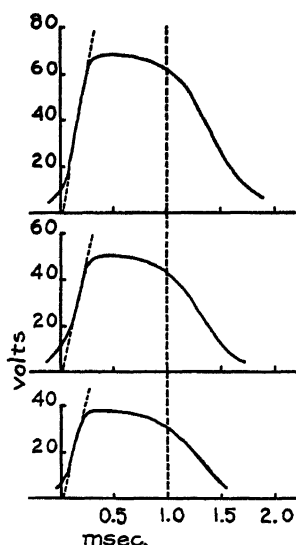


FIG. 10. Tracings from Fig. 9, showing correlation of voltage and time measurements

charges of Hunter's organs, while the regular ones showed the discharges of the main organs alone. But the energy of the anomalous discharges seems to be disproportionate to the size of Hunter's organs, and it appears more likely that they are produced by the same organs as the regular discharges but are modified in some way, perhaps by a lack of synchronization between the discharge in different portions of the organs (5, 9). The traces which were clearly anomalous were not used for the measurement of voltage.

There were 184 usable traces altogether in the six series of observations that were made. Measurements from all but four of these were used in computation. These four were all obtained with one fish, using the oscillograph at high gain and an external resistance of 100 ohms. The peak voltages measured on these traces were much lower than those measured with the same fish and the same external resistance but with the oscillograph at low gain. It seemed probable that the four traces in

question were made with too high a gain for the calibration to be linear and the measurements of voltage were therefore unreliable.

Voltages were measured on each trace at intervals of 0.2 msec., starting at 0.4 msec. from the zero time determined in the manner already described. Measurements were started at 0.4 rather than 0.2 msec. because at the earlier instant they could not be made with any accuracy. This instant falls on the steeply rising part of the trace, where a small shift in the estimated zero time makes a very large change in the measured voltage. At times after 1.4 msec. (after 1.2 with one fish), the voltage was too small for reliable measurement. Thus there were measured generally six values of the voltage during 1 msec. of the discharge. Altogether something more than 1000 measurements were made.

### *Results*

Figs. 11, 12, and 13 show some of the results of three series of observations. The plotted points along each line show, for one value of the time, the values of voltage and current obtained with different values of the external resistance. Each line was fitted to its set of points by the method of least squares. The lines marked (0.4) show measurements at 0.4 msec.; the others show measurements at 1.0 and 1.2 msec.

That the points fit the lines within the uncertainty of the measurement justifies, to this degree of accuracy, the assumption that the electrical behavior of the tissue may be described in terms of E.M.F. and ohmic resistance, not only at the peak of the discharge but at other times as well.

The differences in the slopes of the lines indicate that the resistance of the tissue increases after the peak of the discharge is passed. This agrees with observations made before by another method (9).

It will be noticed that if the three lines are extended to the left of the axis of voltage they come very near to meeting in a point. This is simply explained if it be supposed that the E.M.F. of the tissue and the leakage resistance are constant during the time of the measurements. For it will be recalled that, according to the diagram of Fig. 8, each line is described by the equation,  $V(1 + r/R') = E - Ir$ . In this equation let  $V = E$ . Then  $I = -E/R'$ . Hence if  $E$  and  $R'$  are constant during the discharge and thus the same for all the lines, the point having coordinates  $V = E, I = -E/R'$  will lie on all the lines, which must therefore intersect at this point. It does not seem likely that the near intersection of the lines is an accident resulting from errors of measurement, and the assumption that the E.M.F. and the leakage resistance are at least nearly constant appears to be much the most probable explanation.

The complete results of all six series of measurements appear in Figs. 14, 15, 16, and 17 in a more concise graphical representation obtained as follows. In the equation  $V(1 + r/R') = E - Ir$ , let  $I = 0$  and denote the corresponding value of  $V$  by  $V_0$ . We have then  $V_0(1 + r/R') = E$ . Now use with this

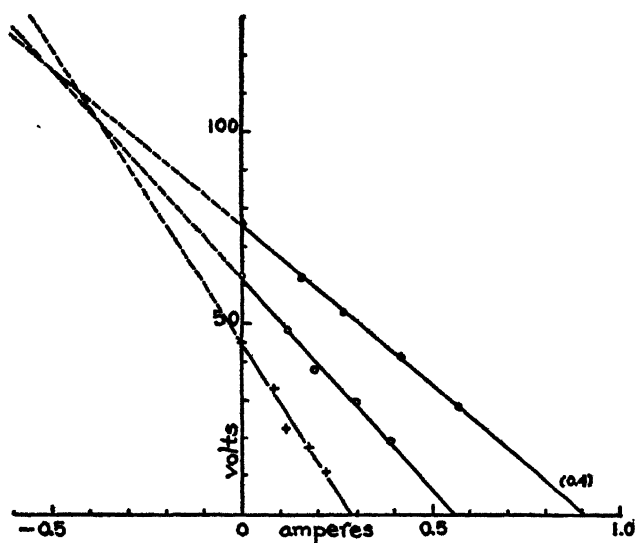


FIG. 11. Evidence of change in resistance of electric tissue and approximate constancy of E.M.F. and leakage resistance during discharge. For explanation see text.

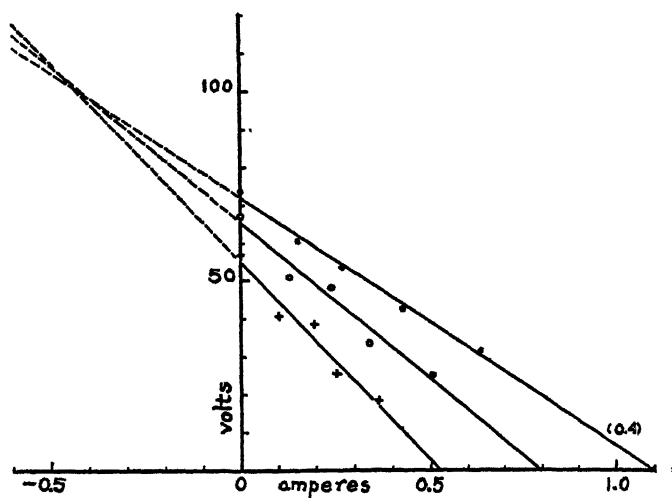


FIG. 12. Resistance and E.M.F. during discharge. Similar to Fig. 11 but another series of observations.

the equation  $1/R' = 1/r + 1/R'$  to eliminate  $r$ , and we find  $V_0 = E - (E/R')r'$ . This is a linear relation between  $V_0$  and  $r'$  if  $E$  and  $R'$  are constants.

In reference to a line on the  $V$ - $I$  graph of Figs. 11, 12, and 13,  $V_0$  is the

intercept of the line with the axis of voltage and  $r'$  is the downward slope of the line. The line is thus completely determined by the values of these two quantities. Therefore if we now employ a new graphical representation with  $V_0$  and  $r'$  as coordinates, a single point of the new representation will correspond to a line on the former one. The three black dots along each line in Figs. 14, 15, 16, and 17 represent measurements at 0.4, 1.0, and 1.2 msec., such as are represented by the three lines in each of Figs. 11, 12, and 13. The lines of Figs. 14 to 17 were fitted to these points by the method of least squares. The white dots in Figs. 14 to 17 represent measurements at 0.6, 0.8, and 1.4 msec.

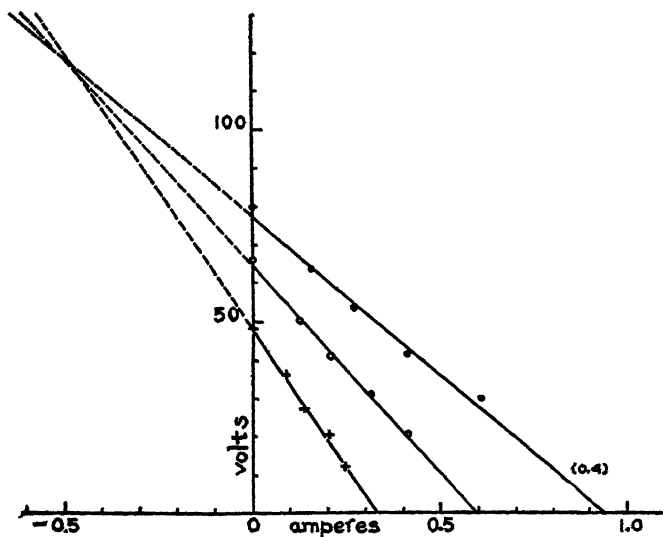


FIG. 13. Resistance and E.M.F. during discharge. Similar to Figs. 11 and 12 but another series of observations.

msec., which were omitted from Figs. 11, 12, and 13 to avoid confusing the figure with too many lines.

In so far as the plotted points of each series fall on a straight line, the measurements support the hypothesis of a constant E.M.F. and leakage resistance. Taking the equation of the line as  $V_0 = E - (E/R')r'$  and letting  $r' = 0$ , we obtain  $V_0 = E$ . Thus the intercept of the line on the axis of  $V_0$  gives the E.M.F. In the same equation, letting  $V_0 = 0$ , we obtain  $r' = R'$ , so that the intercept on the axis of  $r'$  gives the leakage resistance.

The plotted points of Figs. 14 to 17 fall, with three exceptions, quite near the lines. The greatest deviation is 6 per cent and the root-mean-square deviation is 1.8 per cent, the three exceptional points being excluded. The exceptional points all represent measurements at 1.4 msec. and all fall far above the line. On the other hand, two of the graphs have points representing



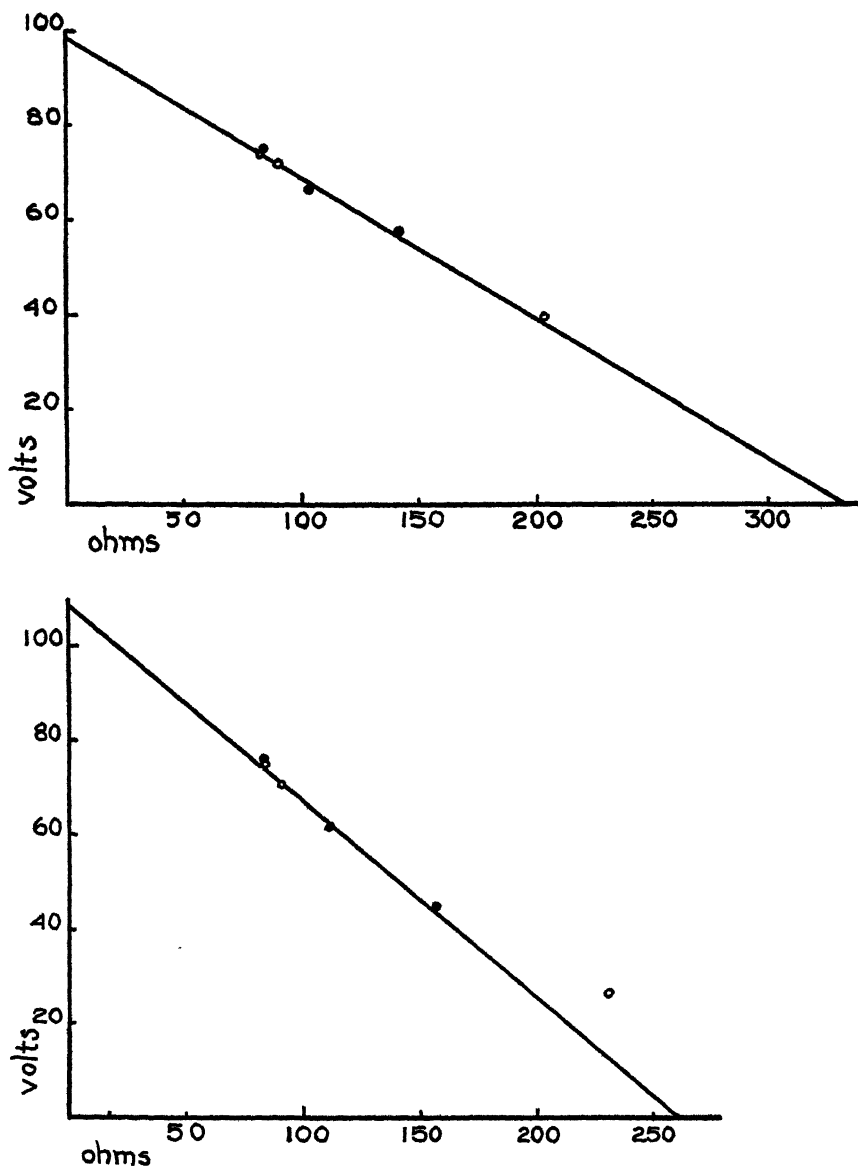


FIG. 14. Resistance and E.M.F. during discharge. Voltage with external circuit open vs. parallel resistance of electric tissue and leakage path. Two series of observations. For explanation see text.

measurements at 1.4 msec. which fall quite near the line, one above and one below. The measurements made on different oscillographic traces at 1.4

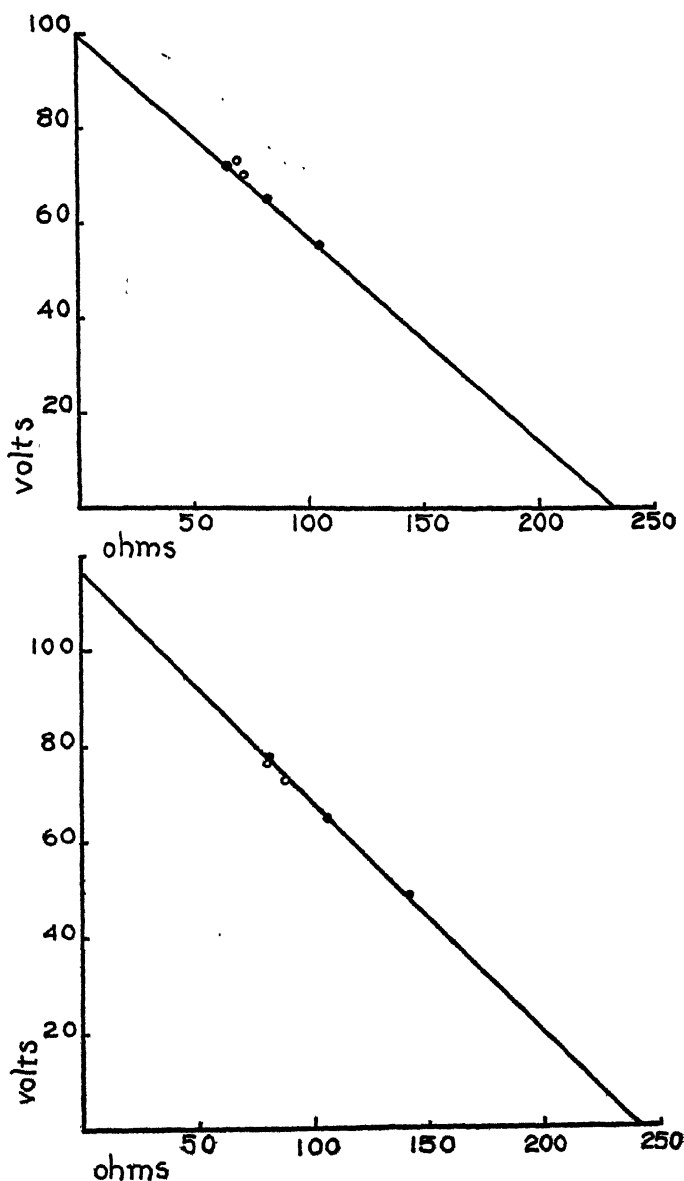


FIG. 15. Resistance and E.M.F. Similar to Fig. 14. Two more series of observations.

msec. show wide variations, and it is possible that the deviations of the exceptional measurements are all accidental. On the other hand, they may be

caused by some electrical characteristic of the tissue of which the diagram of Fig. 8 takes no account and the effect of which becomes important toward the end of the discharge. Our measurements are insufficient to give any safe ground for conjecture on this point.

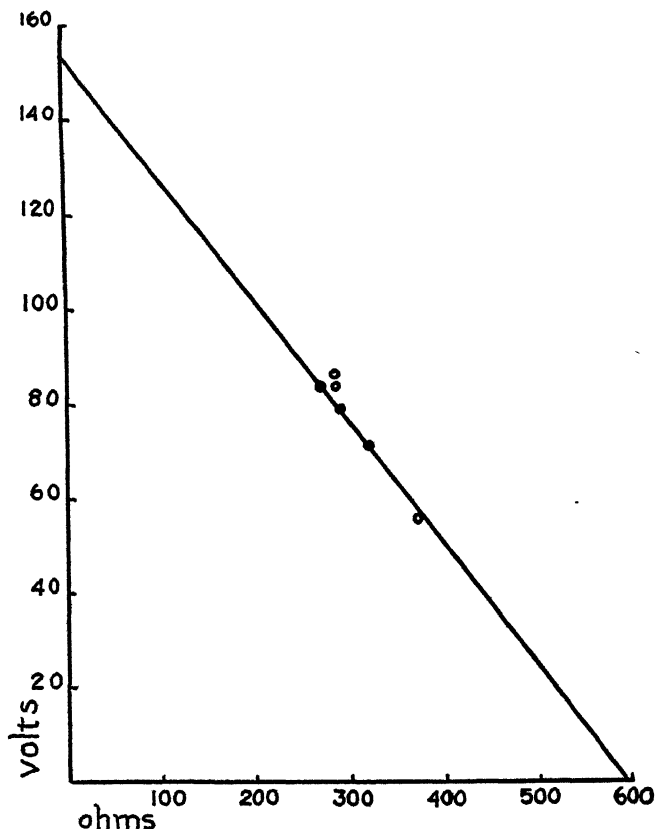


FIG. 16. Resistance and E.M.F. Similar to Figs. 14 and 15. Another series of observations.

In any case it seems clear that the internal resistance of the electric tissue,  $r$  in the equations, varies during the discharge. From the values of  $r'$  and  $R'$  it is easy to calculate, by means of the equation  $1/r' = 1/r + 1/R'$ , the value of  $r$  for each instant of time at which measurements were made. Fig. 18 shows the variation of  $r$  with the time, reckoned from the measurements shown in the upper half of Fig. 14.

Although these measurements extend over only a part of the duration of the discharge and do not include any of the time during which the voltage

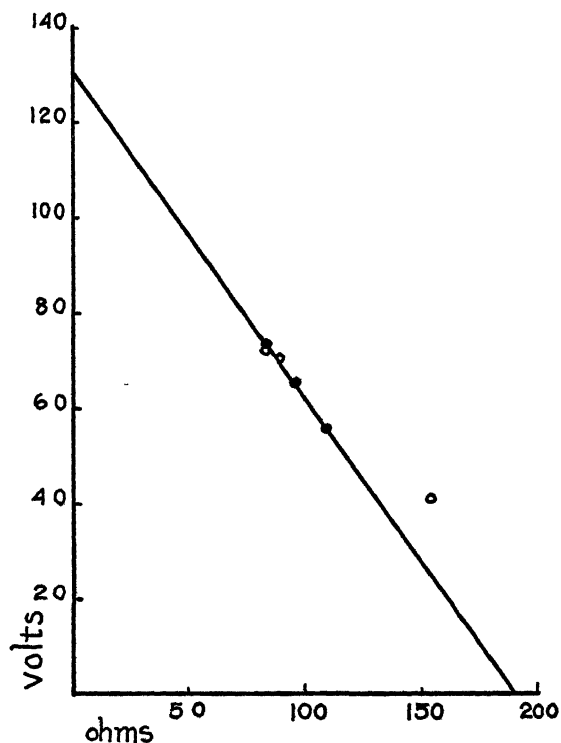


FIG. 17. Resistance and E.M.F. Similar to Figs. 14, 15, and 16. Another series of observations.

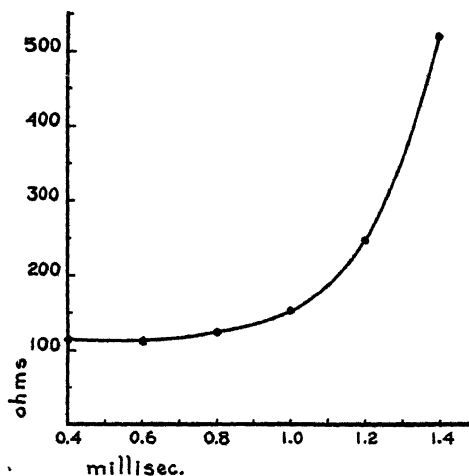


FIG. 18. Variation of internal resistance during discharge. Internal resistance (ohms) vs. time (msec.).

is rising, it seems likely that the variation in resistance is the primary electrical change by which the discharge takes place. According to this view, the E.M.F. is constantly present in the electroplax layer, but, except during the discharge, the resistance of the electroplax boundary is so high that no appreciable current flows and there is thus no appreciable external voltage. The discharge would be started by a very large and rapid drop in the resistance of the electroplax boundary. It would be continued by the resistance remaining briefly at a low value and ended by the resistance increasing to reach again its high initial value.

### *Electric Energy in Relation to Chemical Change*

Since the E.M.F. is apparently constant, at least during the part of the discharge when most of the electric energy is produced, it becomes possible to calculate the total electric energy produced in a single impulse by a given segment of the organs when discharging through a given external resistance. This energy is  $Eq$ , where  $q$  is the charge passing through the segment of the organs in one impulse. Now  $q = \int i dt$ , where  $i$  is the current in the organs, as before,  $t$  is the time and the integration is made over the duration of the impulse. Also  $i = I + I' = V(1/R + 1/R')$ . Hence it follows that  $q = (1/R + 1/R') \int V dt$ . Thus the total electric energy is  $E(1/R + 1/R') \int V dt$ .

The external resistance  $R$  is, of course, known.  $E$  and  $R'$  are the intercepts of the line in the  $V_0 - r'$  graph, as was pointed out earlier. To evaluate the time integral of  $V$ , we have the oscillographic trace, which gives the value of  $V$  at each instant of time. The integration can be performed graphically. The part of the total energy which is delivered to the external resistance  $R$  is  $R \int I^2 dt$  or  $(1/R) \int V^2 dt$ , the integration being made as before over the duration of the impulse. Values of  $V^2$  are computed from the oscillographic trace, and this integration also may be made graphically. The total energy may thus be compared with the energy released externally.

With an external resistance of 100 ohms, the values found for the ratio of the total electric energy to that released externally are 4, 6, 5, 10, 5, 4: average, 6. In an earlier paper the electric energy delivered to an external resistance of 100 ohms was compared with the energy supplied by the breakdown of phosphocreatine and the formation of lactic acid in the electric tissue during the discharge (4). In one series of fifteen experiments it was found on the average that the energy supplied by the breakdown of phosphocreatine was 4.0 times the external electrical energy. In seven of these experiments, the formation of lactic acid was also determined. In the average of these seven, the energy supplied by the breakdown of phosphocreatine was again 4.0 times the external electric energy, and the energy supplied by this and the formation of lactic acid together was 6.5 times the external electric energy. The

chemical energy of both processes, averaged over the seven experiments, was 44 microcal. per gm. and impulse.

The fish used in the present experiments were some of those used in the former ones, and the electrodes were placed in about the same positions on the electric organs. The average external energy per impulse was about the same in the present experiments as in the former. Therefore the two series of experiments are fairly comparable. The fact that in one series the breakdown of phosphocreatine and the formation of lactic acid supplied 6.5 times the external electric energy, while in the other the total electric energy was estimated to be 6 times the external energy makes it very likely that the chemical changes are sources of the electric energy of the discharge.

If it should turn out that the right and left organs discharge separately and not, as we have supposed, together, the comparison of the chemical and electric energies will not be affected. The values found for both energies per gram and impulse will simply have to be doubled and their ratio will thus be left the same. This follows from the manner in which they were determined. The measured chemical changes were those occurring in known masses of tissue in 1600 discharges, and the changes per gram and impulse were reckoned by dividing the changes per gram by 1600. If only one organ discharges at a time, the number of discharges of the organ from which the tissue was taken would have been only 800, and the true values for the changes per impulse would be twice the computed values.

The measured electric energy, on the other hand, was determined for a single impulse and would not have been affected by a mistake in the number of impulses. But it was determined for the whole mass of discharging tissue included between the electrodes. The energy per gram and impulse was estimated by dividing the energy per impulse by the number of grams of tissue. If only one of the two organs discharges at a time, the number of grams would have been half of that assumed, and the true value of the energy per gram and impulse would be twice the computed value.

#### SUMMARY

In the main electric organs of the electric eel, the cross-sectional area, the thickness of the electroplaxes, and certain electrical characteristics of the tissue vary widely between the anterior and posterior ends. However, a transverse layer of the organs one electroplax thick has certain characteristics which are roughly uniform along the organs. These are its volume, its maximum voltage, its maximum current per unit area, and the resistance of unit area at the peak of the discharge.

Measurements of the voltage developed by a segment of the organs across different external resistances at different instants during the discharge are all rather well described by representing the segment, with the adjacent non-

electric tissue, as a simple combination of E.M.F. and ohmic resistance. The internal resistance of the tissue varies during the discharge. Its E.M.F. appears to be practically constant, at least during the greater part of the discharge.

Estimates made of the total electric energy show it about equal to the energy supplied by the decrease of phosphocreatine and the formation of lactic acid.

We wish to thank Dr. David Nachmansohn of the College of Physicians and Surgeons, Columbia University, for his helpful discussion. Dr. A. L. Machado of the School of Medicine, University of Brazil, took part in some of the observations and we regret that he could not remain for the completion of the research. We are grateful also to Mr. Sam Dunton, photographer of the New York Aquarium, for his skill and care in the development of the oscillographic traces, and to Mr. Herman F. Beck and Mr. Joseph Puritch for the construction of some of the apparatus, which was made in the Physical Laboratory of New York University at University Heights.

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## MUSCULAR ACTIVITY AND BUBBLE FORMATION IN ANIMALS DECOMPRESSED TO SIMULATED ALTITUDES\*

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The symptoms of "bends" and "chokes" (sometimes referred to as decompression sickness or aeroembolism) in aviators at high altitude, like those of the same afflictions suffered by divers during ascent to the surface, have often been attributed either directly or indirectly to the presence of nitrogen bubbles in the tissues and fluids of the body (see review by Armstrong, 1939). The "bends" in divers was first shown to be correlated with release of nitrogen in the body by Bert (1878). The few published records of bubble formation in animals decompressed below atmospheric pressure are reviewed by Armstrong (1939), who cites observations of the existence of bubbles by Boyle (1670), Hoppe (1857), and by Hill and Greenwood (1910). Armstrong describes bubble formation in the tissues and blood stream of decompressed goats. Recent evidence indicates not only that "bends" and bubble formation tend to occur together, but also that they commonly are affected in the same way by the same factors. In view of the apparent relationship between these two phenomena the present investigation was undertaken to study factors influencing bubble formation in decompressed animals.

Preliminary work included a search for suitable and convenient biological material for observing bubble formation after decompression to pressures equivalent to high altitudes (60,000 to 70,000 feet).<sup>1</sup> Bubbles were looked for in the blood stream and tissues of animals and in the cytoplasm and yolk of single cells.

Bubbles did not form in single egg cells of the fish *Oryzias* and of the hen, in *Oryzias* or chick embryos, or in small frogs (*Hyla*). They did appear abundantly, however, in the blood stream of the large bullfrog (*Rana catesbiana*)

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<sup>1</sup> At 20°C., at which most of the experiments on cold-blooded animals were conducted, water boils at a pressure equivalent to an altitude of approximately 82,000 feet. At 37°C. water boils at approximately 63,000 feet.



when decompressed to high altitude; e.g., 60,000 feet.<sup>2</sup> Since bullfrogs are convenient animals for experimental purposes, being available in large numbers and able to withstand extreme treatment (dissection, O<sub>2</sub> lack, etc.), extensive experiments were undertaken on this animal. In preliminary experiments it was noticed that during the period of decompression bubbles sometimes appeared in the blood stream immediately after a period of struggle by an incompletely pithed frog. This led to an intensive investigation of the relations between muscular activity and bubble formation.

### *Effect of Muscular Activity on Bubble Formation in Bullfrogs*

A steel decompression chamber 28 × 7 × 7 inches was constructed with a  $\frac{7}{8}$  inch thick plate glass cover which permitted observation of the material during the period of decompression. To permit electrical stimulation of the decompressed frogs, a copper wire grid was placed on the bottom of the chamber and was connected to an outside transformer.

Since preliminary experiments indicated that dissection might possibly favor bubble formation, intact frogs were decompressed to the desired altitude, subjected to varying degrees of muscular activity, and then autopsied after recompression to sea level pressure. Bubbles were looked for in the heart, lungs, arterial, and venous systems. They often became trapped where the renal portal veins enter the kidneys, but could be forced back along these veins into view by pressing the kidneys. In threshold cases in which the simulated altitude was so low that bubbles barely formed (for example, in bullfrogs exercised violently at 20,000 feet), bubbles could be found only in the renal portal veins.

The period of decompression varied from 10 minutes to 1 hour, the usual period being one-half hour. The time necessary to reach the desired altitude and also the time for recompression to sea level was recorded, although apparently within fairly wide limits these two factors do not greatly influence bubble formation in the frog.

The muscular activities of the frogs during the period of decompression were classified into four categories:—

*No Muscular Activity.*—These frogs were not visibly active during the decompression period except for occasional slight breathing movements. Four of the thirty-three frogs in this category were kept motionless by surrounding them with damp towels. The remaining frogs were anesthetized by soaking in a 3 per cent solution of urethane until they were completely relaxed. Breathing movements were very slight, but the heart continued to beat.

*Slight Muscular Activity.*—These frogs were not anesthetized, but the muscular activity during the decompression period was limited to slight movements of the legs and head, and quivering of muscles in various regions. Such movements were induced by shining a strong white light on the frogs.

<sup>2</sup> All altitudes referred to in this paper were simulated in a decompression chamber.

*Extensive Muscular Activity.*—These frogs were stimulated either mechanically or by small electric shocks, inducing such movements as climbing, jumping, and kicking. Only very slight electrical stimulation (2 volts 60 cycle A.C.) was necessary to elicit this degree of activity. In order to rule out any possible effect of the electrical current on bubble formation some of the frogs in this category were stimulated mechanically by attaching one end of a string to a leg and the other end to a rod which could be manipulated from outside the chamber during the decompression period. A sudden jerking of the string would startle the frog into activity.

*Violent Muscular Activity.*—This degree of activity was induced by strong electrical stimulation (10 volts, 60 cycle A.C.) transmitted to the frog through the copper wire grill. The frogs were stimulated for 2 to 3 minute periods at 10 minute intervals during the decompression period. The activity was very violent, involving widespread muscle contraction and tetany, and resulting eventually in complete muscular fatigue.

The presence or absence of bubbles in frogs subjected to these degrees of activity at various altitudes is recorded in Table I, and it is very evident that exercise favors bubble formation.<sup>3</sup> In inactive frogs, bubbles were formed only at 60,000 feet, and even at this altitude only in a small percentage of cases,<sup>4</sup> whereas in violently exercised frogs bubbles were formed at altitudes as low as 20,000 feet. Moreover, bubble formation becomes more profuse as the altitude and degree of activity are increased.

The following experiment provides an especially convincing demonstration of the effect of muscular activity on bubble formation. Using an anesthetized frog with the large veins and arteries exposed and electrodes in contact with the 7th, 8th, and 9th spinal nerves (in the region of the sciatic plexus) or merely on the surface of the hind leg, the animal is decompressed to 50,000 to 60,000 feet. If the frog is quiet no bubbles are observed in the blood vessels. However, if the leg muscles are caused to contract by electrical stimulation (2 to 6 volts), bubbles can be seen almost immediately streaming out of the legs in the femoral and sciatic veins. They are quite large and easily seen with the unaided eye. Bubbles eventually work their way into the heart and in 5 to 10 minutes the entire vascular system may become completely filled with bubbles. If only one leg is stimulated the bubbles come only from that leg. Bubbles have never been observed in the arteries except in cases where they have entered the heart from the venous system and have been pumped into the arteries. It is possible that bubbles form in the arteries, but are not visible

<sup>3</sup> The bubbles formed in violently exercised bullfrogs persist at sea level for about 24 hours. They become invisible at 18 to 20 hours but are readily expanded at this time by decompression.

<sup>4</sup> It is possible that the bubbles found in three inactive frogs at 60,000 feet (Table I) were caused by extremely slight movements that were not readily visible, such as muscle quiverings or breathing movements.

because the direction of blood flow would force them into or through the capillary beds.

E. Newton Harvey (unpublished) later confirmed the finding that muscular activity leads to bubble formation.

TABLE I

*Effect of Muscular Activity during Decompression on Bubble Formation in Bullfrogs*

Simulated altitude	No muscular activity	Slight muscular activity	Extensive muscular activity	Violent muscular activity
ft.				
60,000	----- ----- ----- -++++	+++++ -	+++	+++++
55,000	----	-----	+++++	++
50,000	--	----- -	+++++ +++	++
45,000	----	----	+++	+++++
40,000	----	-----	----	-++++
35,000			--++ -	+++
30,000	--	--	----	+++
25,000			----	-++
20,000	--		--	-++++ -++
15,000	--	--	--	----- -----

Each symbol represents a bullfrog. A + indicates that bubbles formed and a - indicates that bubbles did not form.

Theoretical aspects of bubble formation have been explored by Blinks (unpublished), Dean (1944), and by Harvey *et al.* (unpublished). Their investigations of bubble formation in various fluids have established the fact that following decompression of a mechanically undisturbed fluid, bubbles will appear only if there are small air films ("nuclei") present on particles in the fluid or on the surface of the container. By special methods these surfaces can be cleaned of such nuclei and no bubbles will appear in the fluid on subsequent decompression. However, if the fluid is mechanically agitated the

resulting "negative pressures" are sufficiently great to cause bubbles to form. The ease with which bubbles form is also dependent upon the degree of supersaturation of gases in the fluid.

This concept can perhaps be applied in interpreting the results with the frog. Inspection of Table I indicates that bubble formation depends upon the degree of muscular activity and supersaturation of gases in the blood (as influenced by altitude). The failure of bubbles to form in decompressed, unexercised frogs suggests that there are no bubble nuclei in the blood stream. During muscular contraction, however, negative pressures undoubtedly develop in the blood of the small vessels and capillaries of the muscle, and assuming that these pressure changes are of sufficient magnitude, the conditions necessary for formation of bubbles would thus be fulfilled.

In addition to these physical effects, another concomitant of muscular activity must be considered. It has been clearly demonstrated that  $\text{CO}_2$  greatly facilitates bubble formation (see Harris, Berg, Whitaker, Twitty, and Blinks (1945), who consider at length the effects of  $\text{CO}_2$ ), and since muscular activity results in high local concentrations of  $\text{CO}_2$ , this metabolite undoubtedly plays an important additive rôle in the formation of bubbles in active muscle.

#### *Muscular Activity and Bubble Formation in Rats*

Rats were placed in a small decompression chamber which, after being flushed for about 10 seconds with oxygen, was evacuated rapidly to 50,000 feet. The period of decompression was ordinarily 2 minutes, including the initial 20 seconds required to reach this pressure level. Oxygen was administered throughout the experiment to avoid death or collapse from anoxia. To extend the normal range of activity under decompression, wires for electrical stimulation were attached to the hind limbs above the ankles by strips of cloth soaked in salt solution. When desired, the rats were stimulated intermittently by applying 6 to 13 volts, 60 cycle a.c. The rats employed ranged in weight between 200 and 400 gm., with the majority weighing between 225 to 275 gm. Recompression to atmospheric pressure was rapid in all cases, lasting only 5 to 10 seconds. The rats were then immediately killed for autopsy by breaking the neck with pliers. Standard autopsy procedure, carried out under a dissecting binocular microscope, included examination of the heart, main arteries, and veins, including pulmonary vessels, precaval, postcaval, and hepatic portal systems.

Table II summarizes the results in this series of experiments. In classifying activity of the animals during decompression, four grades or categories were adopted:—

*Essentially Passive.*—The rats remained entirely quiet, or showed only occasional gentle movements.

*Normal Activity.*—The rats moved about freely in the chamber, but showed

no marked alarm or unusual agitation. Animals in this group were not stimulated electrically, although wires to the hind legs were attached in some cases. All other specimens were given handling equivalent to that required for attaching wires, in order to control any possible discrepancy in results which might arise from exercise prior to decompression.

*Moderate Agitation.*—Pronounced activity was evident, of the type ordinarily shown by a rat when frightened or cornered. Such behavior included quick leaps and turns about the chamber, but no activity of an extreme or violent type.

*Violent Agitation.*—Rats in this category displayed the convulsive and highly acrobatic performance characteristic of strong electrical stimulation.

TABLE II  
*Effect of Muscular Exercise on Bubble Formation in Rats during Decompression*

Essentially passive	Normal activity	Moderate agitation	Violent agitation
—	—	(—)	(+). (+)... (+)... (—)
—	—	—	+... (+).. (+).
—	—	+	(+). (+)... (+).
—	—	+	(+). (+). (+)..
—	—	+	(+). (+).. (+)..
—	—	—	
—	—	+	

Rats decompressed to 50,000 feet in 20 seconds for 2 minutes.

O<sub>2</sub> administered during decompression.

+, bubbles; —, no bubbles; ., a few bubbles; .., several bubbles; ... , many bubbles. Parentheses around a plus or minus sign signify the use of electrical stimulation.

This grouping is admittedly somewhat arbitrary, but in all cases, to avoid bias in interpreting results, the animals were classified before autopsy.

Columns 1 (essentially passive) and 2 (normal activity) in Table II are uniformly negative, whereas with one exception all cases in column 4 (violent agitation) show evidence of bubble formation. In column 3 (moderate agitation) an intermediate situation exists, positive and negative cases occurring in approximately equal numbers. It is thus clear that muscular activity favors bubble formation.

The characteristic distribution of bubbles in the blood stream is of particular interest. In no case were bubbles found in the renal veins, hepatic portal system, or pulmonary veins, nor in any artery but the pulmonary artery. The absence of bubbles in these loci suggests that in the present experiments bubble formation was confined to the veins originating in the appendages or body wall. Such bubbles would pass through the right side of the heart and into the pulmonary arteries without obstruction, but would be trapped in smaller vessels

leading to the pulmonary capillaries. This is shown by the fact that through gentle manipulation of the lungs, it is frequently possible to force bubbles backward into the pulmonary arteries, but never into the pulmonary veins. The opaque quality of the lungs prevents direct observation of bubbles in small vessels within the lungs themselves.

Examination of the lymphatics was not included in routine autopsy procedure until the foregoing series of experiments was nearly completed. Subsequently, in similar experiments bubbles have been unmistakably identified in exercised rats, often in considerable number, in the channels leading to the lumbar lymph nodes as well as in the sinuses of these nodes themselves, in the cisterna chyli, and in one instance in the thoracic duct. In two animals the lymphatics proved to be the only locus where bubbles could be found, although usually the presence of bubbles in the lymphatic system is associated with their simultaneous occurrence in the blood stream. In other animals bubbles were identified only in the blood vessels and were not evident in the lymphatics.

Tests indicate that bubble formation does not readily occur in rats at 40,000 feet.<sup>5</sup> Of five cases stimulated into violent activity at this pressure level, all were negative except one animal, in which a few bubbles were found in the pulmonary trunk. Among the four negative cases, one rat was held at 40,000 feet, under strong stimulation, for 15 minutes without effect. Tolerance of rats to altitude was much greater at 40,000 than at 50,000 feet. At the latter barometric pressure, with violent exercise, exhaustion and death ordinarily ensue in rats if decompression is maintained longer than the 2 minute period employed in the present experiments.

It was of interest to note that a colony of docile rats that did not readily engage in extreme or violent activity even with electrical stimulation proved to be relatively bubble-free under decompression, as compared to the more active rats referred to above.

Experiments on decompressed rabbits, young goats, and chickens demonstrated that muscular activity is also conducive to bubble formation in these animals, and Harvey (unpublished) has shown in an extensive series of studies that similar relations obtain in cats.

#### *The Effect of Strong Muscular Activity Prior to Decompression*

Experiments have also been performed to test the effect of strong muscular activity engaged in *before* decompression. Bullfrogs were exercised, to the point of great fatigue, for about 5 minutes on a grid of wires intermittently connected to a source of 2 to 10 volts (60 cycle A.C.). In less than 1 minute

<sup>5</sup> The small size, rapid breathing, and rapid circulation of the rat favor rapid equilibration compared with larger or more sluggish animals, in which similar effects might be expected at lower elevations; the more sluggish bullfrog responds at lower altitudes.

rapid decompression was begun and the frogs were brought to the pressure equivalent of 30,000, 40,000, or 50,000 feet altitude within another 2 minutes. The frogs remained entirely or nearly quiescent under decompression for 30 minutes, after which they were recompressed within 30 seconds, pithed, and dissected to search for bubbles. Unexercised frogs, some of them urethanized, served as controls.

The results are shown in Table III. It is clear that violent muscular exercise immediately *before* decompression favors bubble formation during the ensuing decompression. However, it is less effective than exercise *during* decompression (Table I) since only the latter treatment causes bubbles to form at an altitude as low as 20,000 feet.

TABLE III

*Effect on Bubble Formation in Bullfrogs of Violent Muscular Activity for 5 Minutes Prior to Decompression. Frogs Inactive during Decompression*

Simulated altitude	Violent muscular activity before decompression	No muscular activity
ft.		
50,000	**** **** **+-	----- -----
40,000	**** ***+ *---	----- -----
30,000	+---- -----	----- --

+, bubbles formed; \*, bubbles formed very extensively; —, bubbles did not form.

Experiments were then carried out to determine how long the effects of previous exercise last. Frogs were violently exercised for 5 minutes at atmospheric pressure (sea level) and then, after periods of rest of 1, 30, or 60 minutes, were decompressed to the pressure equivalent of 50,000 feet for 30 minutes before recompression and dissection. Some of those resting 30 and 60 minutes before decompression were urethanized. The results (including non-exercised controls) are shown in Table IV. The controls and the 1 minute cases (extreme left column) in Table IV are taken from Table III. It appears from Table IV that the bubble-producing effect of previous exercise is still present, although somewhat reduced, after 30 minutes; it is largely although not entirely gone after 60 minutes of rest.

At a later date another series of bullfrogs that were considerably smaller than the first was tested and the effect of previous exercise was found to last only 5 to 10 minutes. This difference in the duration of the effect of previous exercise is possibly due to the following factors associated with body size: (1)

degree of mechanical stress, since large frogs have stronger muscles; (2) amount of  $\text{CO}_2$  produced; (3) rate of removal of  $\text{CO}_2$  from the muscles; and (4) rate of equilibration of the frogs on decompression.

In attempting to explain the effect of exercise before decompression we may profitably consider both the rôle of  $\text{CO}_2$  and the possibility that exercise may cause bubbles to form at sea level. It has been shown that  $\text{CO}_2$  greatly facilitates bubble formation (Harris, Berg, Whitaker, Twitty, and Blinks (1945)), and it is produced in high local concentrations by exercise. Time is required to eliminate  $\text{CO}_2$  by ventilation, and the lingering accumulation of  $\text{CO}_2$  could be the cause of the effect, or at least a strong contributory factor. The other possibility mentioned above remains hypothetical. Bubble formation is ordinarily associated with decompression and supersaturation, but Blinks (unpublished) has also produced bubbles in models at sea level (*i.e.* without decompression) by mechanical agitation, and a similar phenomenon is known in

TABLE IV

*Duration of Effect of Exercise Prior to Decompression in Bullfrogs. Decompressed to 50,000 Feet Following Exercise (5 Minutes) and Rest Period of 1, 30, or 60 Minutes*

Duration of rest between exercise and decompression			Non-exercised controls
1 min.	30 min.	60 min.	
****	++++	----	----
****	++--	--++	----
**+-			

+, bubbles formed; \*, bubbles formed very extensively; —, bubbles did not form. The frogs were decompressed for 30 minutes.

cavitation of ship propellers. While such bubbles do not grow, but instead gradually dissolve, they may persist for some time. If small bubbles are produced in similar fashion by muscle action in the frog, they would respond to decompression by expanding greatly to cause the observed results. Immediate dissection of violently exercised frogs failed to reveal the presence of visible bubbles in the blood stream; however, they may have been too small. Both formation of lingering bubbles or nuclei at sea level and accumulation of  $\text{CO}_2$  could be involved.

There appears to be little if any effect of previous exercise on bubble formation in rats. The rat ventilates very effectively with consequences that are discussed in another paper (Harris, Berg, Whitaker, Twitty, and Blinks (1945)). However, Harvey *et al.* (unpublished) have since found that a period of exercise before decompression is effective in producing bubbles in cats.

#### *The Effect of Pre-Oxygenation*

Removal of nitrogen from the body by breathing oxygen before decompression (pre-oxygenation) has been found to reduce the incidence of "bends"



in divers (Behnke, 1937, Behnke and Shaw, 1937) and in aviators (Armstrong, 1939). An experiment was therefore designed to ascertain whether the effect of pre-oxygenation on bubble formation in bullfrogs is the same as its effect on "bends" in man.

Bullfrogs were pre-oxygenated by confinement for 2 to 4 hours in an atmosphere of commercial tank oxygen (Linde) and then immediately decompressed to the pressure equivalents of the altitudes indicated in Table V. The frogs were maintained at altitude for 30 minutes, during which they were intermittently stimulated to violent muscular activity with 2 to 10 volts (60 cycle A.C.) in order to produce conditions favorable for bubble formation. This was

TABLE V

*The Effect of Pre-Oxygenation on Bubble Formation in Bullfrogs*

The frogs in columns 2 and 3 were stimulated to violent muscular activity during decompression. The frogs in column 4 were quiescent throughout.

(1) Simulated altitude	(2) Pre-oxygenated for 2 to 4 hours. Exercised	(3) Not pre-oxygenated (control). Exercised	(4) Pre-oxygenated for 3 hrs. Quiescent
<i>ft.</i>			
40,000	----- -----	+++++ +++++	
50,000	----- -+++		
60,000	----+ ++		----- -----

+, bubbles formed; -, bubbles did not form.

followed by rapid recompression and autopsy. The results are shown in Table V.

In spite of the violent muscular activity, pre-oxygenation prevented bubble formation completely at 40,000 feet (Table V, column 2), while controls not pre-oxygenated all formed bubbles (Table V, column 3). We have already seen (Table I) that violently exercised frogs, not pre-oxygenated, form bubbles at altitudes as low as 20,000 feet. While pre-oxygenation does not prevent bubbles in all exercising frogs at 50,000 to 60,000 feet (Table V, column 2), it considerably reduces the incidence at these high elevations. And when exercise is omitted, pre-oxygenated frogs do not form bubbles even at 60,000 feet (Table V, column 4).

The protective effect of pre-oxygenation is presumably due to removal of nitrogen from the blood stream and body tissues. However, even though pre-oxygenation may reduce the nitrogen tension to such a point that it is no longer in itself able to initiate bubbles in decompressed animals, it is possible

that local high concentrations of  $\text{CO}_2$  in muscles may start bubbles into which any residual nitrogen would then diffuse. The facilitating effect of  $\text{CO}_2$  on the initiation and early growth of bubbles is clearly established (Harris, Berg, Whitaker, Twitty, and Blinks (1945)) and this effect may account for the presence of bubbles in pre-oxygenated frogs exercised at 50,000 to 60,000 feet (Table V).

#### SUMMARY AND CONCLUSIONS

1. Muscular activity during decompression causes bubble formation in the blood of intact bullfrogs. The amount of gas liberated depends on the degrees of muscular activity and supersaturation (as influenced by altitude). In decompressed dissected bullfrogs, bubbles appear in veins leading from active but not from inactive muscles.

2. Muscular activity during decompression similarly causes bubble formation in rats. Bubbles appear in veins coming from muscles, and often in the lymphatic system. Quiescent rats do not form bubbles.

3. Violent muscular activity *before* decompression favors bubble formation in bullfrogs during ensuing decompression, but it is less effective than exercise *during* decompression. The effect persists in large frogs for about an hour.

4. Pre-oxygenation for 2 to 4 hours before decompression reduces the incidence of bubble formation in decompressed bullfrogs. It thus has the same effect on bubble formation in bullfrogs as it does on the "bends" in man. The effect is presumably due to removal of nitrogen.

5. Possible mechanisms by which muscular activity causes bubble formation are discussed. The effects of mechanical agitation and of metabolic  $\text{CO}_2$  are considered to be the dominant factors.

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# CARBON DIOXIDE AS A FACILITATING AGENT IN THE INITIATION AND GROWTH OF BUBBLES IN ANIMALS DECOMPRESSED TO SIMULATED ALTITUDES\*

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Aviators, like divers, may be subject to decompression sickness following a marked drop in external barometric pressure of sufficient duration. In divers, the symptoms, known as the "bends" and the "chokes," were associated by Bert (1878) and by Boycott, Damant, and Haldane (1908), on the basis of animal experiments, with the liberation of dissolved nitrogen in the form of bubbles in the blood and tissues. Important applications of this principle have been made by Behnke and associates (summary, Behnke, 1942) in practical operations. Until recently, however, little evidence was available regarding bubble formation in animals decompressed from sea level to simulated altitudes (*i.e.*, to pressures of less than one atmosphere). Isolated early observations were made by Robert Boyle (1670), Hoppe (1857), and by Hill and Greenwood (1910), while the outstanding modern work antedating the war is that of Armstrong (1939). Using goats, Armstrong found bubbles in blood and tissues on decompression to simulated altitudes of 40,000 feet; smaller animals remained bubble-free under similar conditions.

Recently we have completed an extensive series of experiments on bubble formation in animals at reduced pressures, the results of which are reported in a separate communication (Whitaker, Blinks, Berg, Twitty, and Harris (1945)). The most important aspect of this work was the finding that muscular exercise in decompressed animals greatly favors bubble formation. Bubbles may appear in small animals (bullfrogs, rats) as well as in larger forms (rabbits, goats) following induced or spontaneous activity at simulated altitudes; in quiescent animals bubbles are absent. E. Newton Harvey and coworkers (unpublished) subsequently confirmed these results, using bullfrogs and cats as experimental animals.

The detailed mechanism of action of exercise on bubble formation has been the subject of further study. Blinks (unpublished), Dean (1944), and Harvey

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*et al.* (unpublished) have analyzed the rôle of purely physical factors (turbulence and other effects) which are involved in the action of exercise. They have shown that mechanical agitation causes bubble formation under conditions of supersaturation. We have studied the influence of certain metabolites resulting from muscular activity, particularly carbon dioxide. The possibility was early recognized that high concentrations of  $\text{CO}_2$ , present locally in the muscles during muscular exercise, might affect bubble development in these sites. This idea has been confirmed by numerous experiments, the results of which are reported in the following sections.

*Materials and Methods.*—For purposes of decompression, a small steel chamber,  $7 \times 7 \times 28$  inches, open at the top, was used. The top was covered with plate glass to permit direct observation of animals under reduced pressures. By means of a small pump and reserve vacuum tank, the pressure could be lowered to a simulated altitude<sup>1</sup> of 50,000 feet in approximately 45 seconds. Pressures within the chamber were measured with a mercury manometer, calibrated in terms of equivalent altitudes. In experiments involving exercise, frogs were placed on a wire grid and stimulated intermittently with 5 to 25 volts, 60 cycle a.c. For rats, this purpose was accomplished by applying electrodes, wet with saline, to the hind limbs and stimulating similarly.

#### *Bubble Formation in Dead Animals*

We were encouraged to investigate the rôle of  $\text{CO}_2$  in bubble formation by the suggestive observation that in certain cases dead animals, if decompressed to 45,000 to 50,000 feet, exhibited widespread and profuse bubble formation in the vascular system. This finding was particularly interesting because of the absence of muscular activity or turbulence of the blood in dead animals. The first experiments of this type were performed with rats, killed by breaking the neck (without external rupture). The animals were then allowed to stand at atmospheric pressure and room temperature for 5 to 10 minutes longer to assure complete cessation of breathing and heart action. Rapid decompression to 50,000 feet followed, the rats remaining at altitude for 10 minutes. At the end of this time the animals were returned to atmospheric pressure and submitted to careful autopsy under a dissecting binocular. In typical cases the heart, arteries, and veins all contained large numbers of bubbles. With some variation between individual rats, the distribution of bubbles extended to all vascular branches, including all chambers of the heart, pulmonary arteries and veins, aorta and branches, as well as the precaval, postcaval, and hepatic portal systems. Often the lymphatic system, particularly the lumbar lymph nodes and cisterna chyli, also contained bubbles.

The possibility was originally considered that these bubbles found at autopsy

<sup>1</sup> It should be understood that all altitude levels referred to in this paper are *simulated* altitudes (*i.e.* equivalent barometric pressures).

were introduced during the decompression through rupture of capillaries, either by expanding intestinal gases or in the lungs. In order to determine the site of origin of these bubbles, therefore, a number of dead rats were dissected open with minimal damage to blood vessels and decompressed. The animals were prepared so as to permit direct or microscopic observation at altitude of all the main vessels of the circulation. Observation in these cases revealed that bubbles were not apparent for a short interval after reaching the simulated altitude (50,000 feet); then suddenly a stream of bubbles appeared, coming from a small branch into a larger vessel, followed by bubbles arising similarly in other loci. Local fluctuations in the rate of bubble formation led to an irregular ebb and flow of bubbles in the vessels. In a short time the larger vessels and heart contained a froth or became entirely filled with gas from the coalescence of massive accumulations of bubbles. The pressure from this expanding gas phase resulted in forcing the bubbles throughout the vascular system, in arteries as well as veins. No bubbles were seen to arise in larger vessels, but invariably appeared to come from the smallest branches, predominantly from muscles of the legs and back. In no case did the primary source of bubbles involve the vessels from either the lungs or the digestive tract.

In order to test this point further, additional experiments were performed with dissected dead rats, in which the postcava and dorsal aorta were clamped off just anterior to the diaphragm. Several of these animals on decompression had great numbers of bubbles posterior to the clamps before any bubbles had yet appeared in the heart or lung regions, thus eliminating in these cases the lungs as a source. Likewise, when clamps were placed on postcava and aorta posterior to vessels supplying the digestive tract, bubbling occurred abundantly in the isolated posterior region. These observations indicate that bubbles in dead rats are not derived by "leaking" into the vascular system, but come from small vessels, deep within the tissues, and most commonly from muscular regions.

The question also arose whether bubbling in the decompressed dead rats might be due to some slight muscular activity (*e.g.*, peristalsis) still persisting after breathing and heart action had ceased. To check this point, six rats were killed as before and allowed to stand for 4 hours at room temperature. At the end of this time the animals were in rigor mortis and the body was cool. On subsequent decompression and autopsy, three rats contained great numbers of bubbles throughout the circulatory system, indicating that the cause of bubbling in these animals could not be attributed to lingering muscular activity.

Other experiments showed that the phenomenon of bubble formation in decompressed dead animals was not confined to rats. Several rabbits killed and decompressed according to the same procedure showed a similar widespread occurrence of bubbles at autopsy. It is difficult to perform comparable experiments with bullfrogs, since the heart continues to function even after the cen-

tral nervous system is destroyed. However, a number of frogs which had died from "red-leg" were used for this purpose. These were decompressed, 24 to 48 hours after death, to 50,000 feet for 10 minutes. Of fifteen frogs treated in this manner, bubbles occurred in eight.

In general, while the majority of decompressed dead animals contained bubbles, a certain number of individuals remained bubble-free. A rather sharp difference existed between the two groups of animals: typically, bubbling occurred profusely, spreading throughout the body, or not at all. Other experiments were performed to study this variability further. Preliminary work revealed no simple correlation of size or amount of fat with occurrence of bubbles in rats when killed and decompressed, although a trend existed toward a somewhat higher frequency of bubble formation in large, well fed animals.

Attention was also directed to the method of killing used in the first experiments (breaking the neck) to see if any factor inherent in this procedure was responsible for the variability noted. In some cases rats were killed by simple manual suffocation, to minimize the effect of trauma and attendant possibility of admitting air through injured tissues. This treatment did not, however, alter the incidence of bubbles found at autopsy, done after decompression. Another group of animals was killed by acute anoxia at simulated altitudes. These animals were decompressed in air, while still alive, to 50,000 feet for 4 minutes. Death occurred within a minute or two from oxygen lack. After the 4 minute period, the rats were recompressed and immediately autopsied. As before, the greater number, but not all, of these rats contained bubbles. In addition, the last mentioned experiments suggest that under certain circumstances the characteristic capacity noted for bubble formation may be present almost immediately after death, without the necessity of any intervening latent period. The effects of pure nitrogen and illuminating gas as lethal agents were also investigated and gave results similar to those already described.

Since all of the above mentioned methods of killing involve violent or paroxysmal muscular activity as a terminal phase, experiments were designed to determine whether this was an indispensable condition for bubble formation after death. A number of rats were given lethal doses of nembutal (100 mg.) intraperitoneally, with a minimum of disturbance to the animals. Several of these were entirely quiescent during and after injection. Fifteen to 30 minutes after breathing and heart action had stopped, the animals were decompressed to 50,000 feet for 10 minutes. At autopsy a number of rats, including the quiescent animals, contained bubbles. It is evident, therefore, that extensive muscular activity just prior to death is not a necessary factor for bubble formation in dead rats. The modifying effect of prolonged violent exercise before death will be considered later.

Electrocution was found to be the most effective lethal agent, from the standpoint of a high incidence of bubble formation on subsequent decom-

pression. At first, electrodes wet with saline were attached diagonally to one front and one hind leg and current (110 volts, 60 cycle A.C.) applied for 1 minute. Later the animals were merely dropped into a saline bath into which the two electrodes had been immersed. As a control procedure for this method, six rats were left in the circuit for 30 minutes and then immediately autopsied (no decompression). No bubbles were found on careful search in these animals, indicating that electrocution *per se* is not a cause of bubble formation. Using this method, twenty rats were killed and subjected to decompression, of which only three failed to give bubbles. These results are summarized in Table I together with those obtained with other lethal agents. It is evident that a higher percentage of positive cases is obtained with electrocution, compared to other methods. This difference may conceivably be connected indirectly

TABLE I  
*Bubble Formation in Decompressed Dead Rats*

Cause of death	Time between death and decompression	Duration of decom- pression	Simulated altitude	Rats with bubbles present	Rats with no bubbles
	<i>min.</i>	<i>min.</i>	<i>ft.</i>		
Broken neck.....	5-10	15	50,000	9	5
Manual suffocation.....	10	15	50,000	5	2
Anoxia (at altitude).....	0	4	50,000	6	3
Nitrogen.....	5	15	45,000	3	2
Illuminating gas.....	10	4	50,000	1	1
Nembutal.....	15-30	10	50,000	14	6
Electrocution, 110 v.A.C.....	10-30	10	50,000	17	3

with the demonstrable heating effect caused by passage of current through the tissues. Such heating might tend to delay the rate of cooling in the body after death. In support of this view is the fact that if rats are killed by nembutal and maintained in an incubator at 37°C. during the interval before decompression, the incidence of bubble formation tends to increase on later low pressure treatment. Furthermore, if rats are anesthetized and then killed by cold treatment without freezing, and are decompressed in ice water, bubble formation does not occur. If, however, rats killed by cold are warmed to 37°C. in an incubator for 45 minutes before decompression, bubbles form as usual with low pressure treatment. Hence it seems possible that the lower percentage of positive results in dead rats with lethal agents other than electrocution may be due to the cooling process after death, with consequent greater solubility of dissolved gases.

While temperature may constitute a cause for a certain number of negative results in the dead animals, it is not a complete explanation. Both in rats killed by electrocution and those killed by nembutal but maintained at 37°C.,



negative cases were still apparent. This finding, together with the essentially all-or-none character of bubble occurrence in the dead animals, suggests a threshold phenomenon, possibly in relation to the degree of supersaturation of dissolved gases in the body. This idea is supported by evidence from rats decompressed from high barometric pressures to sea level (Harris, Berg, Whitaker, and Twitty (1945)) where bubbles may form in quiescent living rats without any muscular exercise, if the degree of decompression (which influences supersaturation) is 60 pounds per square inch or greater.

It seems unlikely that variation in  $N_2$  supersaturation between individual dead rats could account for the occasional negative results in question, since all animals were equilibrated similarly with air throughout the experiments. On the other hand the amount of dissolved free  $CO_2$  in blood and tissues probably reaches high levels in dead animals, and might be subject to a considerable variation between individual rats. After death, the normal  $CO_2$  tension is augmented not only by residual cellular respiration, the products of which are not removed, but also still further by anaerobic glycolysis. The latter process involves formation of lactic acid which reacts with bicarbonates in blood and tissue fluids to liberate additional free  $CO_2$ .

The extent of anaerobic glycolysis in dead animals is indicated by Voegtlin's studies (1933) with a glass electrode on pH changes in muscle, in which post mortem values as low as 6.2 were recorded. That anaerobic glycolysis may markedly increase the amount of free  $CO_2$  is indicated further by experiments *in vitro*. Rat blood in a clean test tube will not bubble under decompression to 50,000 feet unless given strong mechanical agitation. If lactic acid is added in physiologically possible concentrations (0.4 per cent), bubbling likewise will not occur at simulated altitudes if the tube is undisturbed, but a slight tap will induce violent frothing.

Animal experiments were also devised to test the importance of  $CO_2$  liberated by lactate formation for bubble development under decompression. For this purpose rats were used in which anaerobic glycolysis had been largely inhibited. The experimental animals were given lethal doses of sodium iodoacetate (370 to 550 mg.) intraperitoneally; a control series received lethal doses of nembutal instead. At the time of death, both experimental and control animals were placed in an incubator at  $37^\circ C$ . to eliminate any possible effects of cooling before decompression. After intervals varying from 10 minutes to 2 hours, the rats were decompressed to 50,000 feet for 10 minutes. Table II summarizes the results obtained at autopsy. It is evident that the reduction or inhibition of anaerobic glycolysis by iodoacetate poisoning is correlated with a definitely lower incidence and extent of bubble formation in decompressed dead rats.

Additional information regarding the rôle of  $CO_2$  liberated from bicarbonates by lactic acid is suggested by experiments dealing with the effect of strong exercise just prior to death on bubble formation in dead rats. These animals,

through electrical stimulation (5 to 25 volts, 60 cycle, A.C.) and associated spontaneous reactions, underwent 15 minutes of violent and maximal activity, resulting in a state of exhaustion. The rats were then immediately killed (electrocution) and after 30 minutes decompressed to 50,000 feet for 10 minutes. Control animals were electrocuted without previous exercise and decompressed similarly. Table III presents the results obtained on the two groups, and indicates a definite trend toward protection against bubble formation in the pre-

TABLE II  
*Effect of Sodium Iodoacetate on Bubble Formation in Decompressed Dead Rats*

	Lethal agent	Time between death and decompression	Bubbles at autopsy		
			None	Very few	Many
Experimental series.....	NaIA, (370-550 mg.)	10 min. to 2 hrs.	5	6	0
Control series.....	Nembutal (100 mg.)	10 min. to 1 hr.	2	1	6

Experimental and control animals maintained at 37°C. before decompression to 50,000 feet for 10 minutes.

TABLE III  
*Effect of Violent Muscular Exercise, Just Prior to Death, on Bubble Formation in Decompressed Dead Rats*

Previous exercise	Bubbles at autopsy		
	No bubbles	Bubbles in venous system and right side of heart only	Bubbles in arteries, veins, and heart
Violent.....	4	6	0
No exercise.....	1	0	11

Rats stimulated intermittently for 15 minutes (5 to 25 volts, 60 cycle A.C.) before death by electrocution. After 30 minutes, decompressed to 50,000 feet for 10 min.

exercised rats. This finding on the surface appears paradoxical in view of the predisposing effect of pre-exercise on bubble formation in living bullfrogs (Whitaker, Blinks, Berg, Twitty, and Harris (1945)). However, it is possible that the results obtained here with rats may be attributed to the extreme degree of exercise used. In this connection, the studies reviewed by Dill (1936) on blood lactate and bicarbonate during muscular exercise in man are of interest. He reports that under conditions of mild or moderate exercise, no appreciable rise in blood lactate is observed. After extremely violent exercise, however, large quantities of lactate are found in the blood and a drop of bicarbonates to low levels is observed. The latter process is apparently due to liberation of free

CO<sub>2</sub> by lactate formation, which is lost *via* the lungs, and to the severe hyperpnea and increased loss of CO<sub>2</sub> from this source *per se*. It seems probable that these factors may operate similarly in the rat, especially in view of the small size and consequent rapid respiratory turnover. Thus, it is possible that in the present experiments, the extremely violent exercise and attendant hyperventilation may have so reduced the bicarbonate reserve that anaerobic glycolysis after death did not liberate sufficient CO<sub>2</sub> to produce the usual effect on bubble development. As seen in Table III, this view is supported by the characteristic distribution of bubbles in the positive cases among the experimental animals: bubbles occurred only on the venous side of the circulation, where the CO<sub>2</sub> tension is highest.

The sluggish bullfrog, on the other hand, has by comparison with the rat a highly inefficient lung and ventilation rate, so that the free CO<sub>2</sub> produced in muscular exercise may not have been eliminated to any great extent before decompression, and could therefore facilitate bubble formation at altitude. In living mammals (cats) Harvey (unpublished) have shown that pre-exercise facilitates bubble formation on later decompression, a result which harmonizes well with our earlier findings on bullfrogs, but differs from our preliminary results in a few experiments on living rats, where a protective effect was suggested. In view of Dill's statement, mentioned above, that a drop in bicarbonate reserve occurs in severe but not in moderate exercise, such variability might conceivably result if somewhat different levels of muscular activity were involved in the two cases. Also the cat, being larger than the rat, may possibly eliminate CO<sub>2</sub> less rapidly than the rat (as in the frog).

Considering the complex group of factors involved, it is not unreasonable to expect a certain random variation in the final level of CO<sub>2</sub> attained in dead rats, although a moderate increase could result in a condition of supersaturation with respect to CO<sub>2</sub> when the animals were decompressed. For example, if the normal venous CO<sub>2</sub> tension is roughly approximated at 5 per cent (38 mm.), then, to select an arbitrary figure, a mere threefold increase in the dead animals could result in a CO<sub>2</sub> level of 114 mm. At 50,000 feet (87 mm. barometric pressure), from Henry's law that solubility is a function of partial pressures, this CO<sub>2</sub> figure represents a state of supersaturation in the dead rats. This condition follows from the fact that the dead animal is not a rigid object and the pressure within bubbles inside the body will be substantially identical with external barometric pressure. Thus at 50,000 feet (87 mm. barometric pressure) a bubble in the blood stream would have an internal pressure of approximately 87 mm. and would grow constantly from CO<sub>2</sub> alone, if the CO<sub>2</sub> tension in its surroundings exceeded this level, as in the example given above. Nitrogen would likewise augment bubble growth, for similar reasons.

Furthermore, CO<sub>2</sub> under supersaturation, when compared to N<sub>2</sub> under similar conditions, exerts an effect all out of proportion to the degree of super-

saturation involved. In experiments with models, Blinks (unpublished) has shown that bubbles develop more readily in water saturated with  $\text{CO}_2$  than with air. We have likewise studied this effect *in vitro* by saturating water in clean test tubes with one atmosphere of  $\text{CO}_2$  and  $\text{N}_2$  respectively, and decompressing the tubes to the equivalent of 50,000 feet. Bubbles form with much less mechanical agitation in the  $\text{CO}_2$  water and grow in size at a higher relative rate. These effects may be related to the relatively high solubility of  $\text{CO}_2$ , with consequent large numbers of dissolved molecules. Thus even a relatively small degree of  $\text{CO}_2$  supersaturation in the dead animals might be significant in bubble formation.

With regard to the mode of action of  $\text{CO}_2$  on bubble formation in dead animals, one possible explanation is as follows: The experiments *in vitro* mentioned above, with  $\text{CO}_2$ -saturated water, and with lactic acid added to rat blood, suggest that if the concentration of supersaturated gas molecules is greatly increased, there is a corresponding marked reduction in the degree of mechanical agitation needed to induce bubble formation. It is therefore possible that while a high level of  $\text{CO}_2$  in the dead animal does not in itself directly initiate bubble formation, it may predispose in this direction to the point where the very slight mechanical disturbances unavoidably present in decompression of the dead animals (mere expansion of the body under lowered pressure) may be sufficient to produce bubbles. In occasional animals with a lesser accumulation of  $\text{CO}_2$  these slight strains might prove insufficient to initiate bubble formation, thus accounting for the threshold nature of the phenomenon.

The reasoning given above may also provide an explanation for certain results obtained by applying a tourniquet to the legs of decompressed animals. It has been found with young goats (Blinks and Reed, to be published) that considerably less muscular activity is required to produce bubbles in a leg if a tourniquet is used. The  $\text{CO}_2$  content is undoubtedly increased by the tourniquet, and this may explain the reduced threshold of activity for bubble formation.

#### *Effect of $\text{CO}_2$ Administration on Bubble Formation*

It should be emphasized that the rôle of  $\text{CO}_2$  as a means of facilitating bubble formation is in no way restricted to dead animals or extreme pathological conditions. The prime purpose of the analytical work presented thus far lies in revealing the importance of  $\text{CO}_2$  for bubble formation in the living, intact animal. As already pointed out, the data thus obtained support the idea that the favoring effect of pre-exercise on bubble formation in decompressed bullfrogs can be explained by the accumulation of  $\text{CO}_2$  in the body. We have in addition put this theory to a more direct test by treating living frogs with high concentrations of  $\text{CO}_2$ , in an effort to simulate the effects of pre-exercise on bubble formation. Bullfrogs were placed in an atmosphere of 60 to 70 per

cent CO<sub>2</sub> (balance air) for 2 to 3 hours. Following this treatment, which the frogs tolerate fairly well, they were decompressed to various pressure levels for 30 minutes. At altitude some remained quiescent; others underwent varying degrees of spontaneous activity. Still others, previously urethanized, were completely inactive. As controls, frogs not treated with CO<sub>2</sub> were decompressed similarly. The results are shown in Table IV. At 60,000 feet, and without any appreciable muscular activity (quiescent or urethanized frogs), bubbles formed in a majority of the CO<sub>2</sub>-treated animals, while only three out of eighteen frogs in the control group contained bubbles. Bubble formation in many of the CO<sub>2</sub>-treated frogs was very extensive and almost explosive in

TABLE IV

*Bubble Formation in Decompressed Frogs Previously Treated with CO<sub>2</sub> (Compared with untreated Controls Decompressed Similarly)*

Pre-treatment	Altitude	Activity	Treated animals		Controls decompressed similarly, without CO <sub>2</sub> pre-treatment	
			Bubbles present	No bubbles	Bubbles present	No bubbles
<i>per cent CO<sub>2</sub></i>	<i>ft.</i>					
60-70	60,000	None (urethanized)	6	3	3	15
60-70	50,000	None (2 urethanized)	0	3	0	2
60-70	50,000	Slight (spontaneous)	6	0	0	7
60-70	15,000	Violent (stimulated)	2	3	0	5
25	50,000	Slight (spontaneous)	1	3	0	5
25	50,000	Moderate	2	0	7	0

Experimental animals placed in CO<sub>2</sub> mixtures for 1.5 to 3.5 hours before decompression. Duration of decompression 2 to 10 minutes.

character, beginning abruptly after a short latent period at altitude, and causing rapid and pronounced swelling of the body. Such phenomena did not occur in control animals even when bubbles were formed. At 50,000 feet, the differences between experimental and control groups were even more clear cut; here bubbles formed in CO<sub>2</sub>-treated frogs with slight muscular activity, although the same degree of exercise did not result in bubble formation in any of the untreated controls. It is evident that direct administration of CO<sub>2</sub> to living frogs facilitates bubble formation on subsequent decompression, just as does previous exercise at sea level. However, high concentrations of CO<sub>2</sub> are required to produce this effect; a few experiments showed that 25 per cent CO<sub>2</sub> has only a slight effect on subsequent bubble formation (Table IV). This may in part reflect the difficulty of reproducing the local CO<sub>2</sub> picture in capillaries and small vessels by diffuse external application. The CO<sub>2</sub> concentration may be very high locally in the normal exercised animal.

CO<sub>2</sub> treatment of frogs, besides facilitating bubble formation at relatively

high altitudes, also causes a lowering of the threshold altitude at which bubbles will form. Bubbles ordinarily will not form (or at least cannot be observed) in normal bullfrogs violently exercised at a pressure equivalent to 15,000 feet. However, bubbles will appear at this same altitude in frogs which have been in a 70 to 80 per cent CO<sub>2</sub> atmosphere for 3 to 4 hours and then violently exercised at 15,000 feet (see Table V). In two frogs bubbles appeared as low as 10,000

TABLE V

*Effect of CO<sub>2</sub> Treatment on Threshold Altitude for Bubble Formation in Bullfrogs (R. catesbiana)*

Altitude	Pre-treatment	Activity	Autopsy	
			Bubbles present	Bubbles absent
<i>ft.</i>	<i>per cent CO<sub>2</sub></i>			
15,000	60-70	Violent	4	3
10,000	60-70	Violent	2	3
5,000-7,000	60-70	Violent	0	6
15,000	None (controls)	Violent	1	6

Experimental frogs in CO<sub>2</sub> 3 to 4 hours before decompression.

TABLE VI

*Effect of CO<sub>2</sub> Treatment on Threshold Altitude for Bubble Formation in Grass Frogs (R. pipiens)*

Altitude	Pre-treatment	Activity	Autopsy	
			Bubbles present	Bubbles absent
<i>ft.</i>	<i>per cent CO<sub>2</sub></i>			
25,000-30,000	60-70	Violent	3	1
20,000	60-70	Violent	3	6
10,000-15,000	60-70	Violent	0	8
70,000	None (controls)	Violent	5	0
60,000	None (controls)	Violent	1	6

Experimental frogs placed in CO<sub>2</sub> mixtures for 3 to 4 hours before decompression.

feet. There is an optimum concentration of CO<sub>2</sub> for demonstrating this phenomenon. Too much has a narcotic effect so that the frogs will not exercise violently, whereas if too little CO<sub>2</sub> is administered the facilitating effect disappears. Lowering of the threshold altitude for bubble formation is even more striking in grass frogs (*Rana pipiens*), which are smaller than bullfrogs. Ordinarily bubbles will not form in grass frogs, even under violent exercise at a pressure equivalent to 60,000 feet. But if these frogs are placed for 3 to 4 hours in 70 to 80 per cent CO<sub>2</sub>, bubbles will appear on violent exercise at a pressure equivalent to 20,000 feet (Table VI).

The ratio of minimum barometric pressures at which bubbles may be formed in normal frogs of these two species is as follows.

$$\frac{\text{Bullfrogs}}{\text{Grass frog}} = \frac{349}{54} = 6.5$$

Thus the threshold pressure for bubble formation in the bullfrog is over 6 times that which is required for grass frogs. In frogs pre-treated with CO<sub>2</sub> this ratio becomes

$$\frac{\text{Bullfrog}}{\text{Grass frog}} = \frac{429}{349} = 1.2$$

Thus the effect of CO<sub>2</sub> is to reduce the threshold barometric pressures required for bubble formation in these frogs nearly to a common level. In general, it is more difficult to obtain bubbles in small frogs than in large ones. CO<sub>2</sub> treatment tends to make this size difference disappear, a fact which may throw some light on why the original difference exists. Assuming that CO<sub>2</sub> is important for bubble initiation and that it has to reach a certain molecular concentration to be effective, then it may be that small frogs do not form bubbles readily because the CO<sub>2</sub> diffuses out of their smaller muscles much faster than out of the larger muscles of bullfrogs. Thus a threshold concentration of CO<sub>2</sub> would not be reached as easily in small muscles and small animals as in large ones.

The small residual difference in threshold altitudes for bubble formation in these frogs after treatment with CO<sub>2</sub> may be due to a difference in the degree of mechanical disturbance occurring during muscular activity in the two sizes of muscles, since the muscular activity of bullfrogs is more powerful than that of grass frogs. This idea is supported by negative results obtained in attempts to induce bubble formation in small flaps of exposed muscle in large frogs, where mechanical agitation would be less, due to the relatively weak contractions. However, the relatively high outward diffusion of CO<sub>2</sub> from these flaps may also be involved.

#### *Gas Analysis of Bubbles*

Another and even more direct approach to the importance of CO<sub>2</sub> in bubble formation lies in gas analysis of the bubbles themselves, formed under various conditions. For this purpose a new procedure has been developed by one of us (Berg, to be published) for the estimation of CO<sub>2</sub> and O<sub>2</sub> in small bubbles. The method adopted permits analysis of bubbles 0.4 to 1.5 mm<sup>3</sup>. in volume. Briefly, it involves the use of a long piece of capillary tubing, of uniform bore, with a bell-shaped enlargement on one end and a piece of rubber tubing and screw clamp, to exert suction, on the other end. Bubbles placed in the bell with a pipette may be drawn in and out of the fine bore for measurement before and after reaction with KOH (to determine CO<sub>2</sub>) and pyrogallol (to determine O<sub>2</sub>).

The method for obtaining the gas sample from the blood stream is as follows: a bubble of sufficient size for analysis is located in a vein, which is then cut. As the bubbles come out, one is picked up in a pipette filled with saturated LiCl (this reagent is used to reduce diffusion of gas in or out of the bubble) and transferred to a pool of LiCl solution to free it of blood and then is introduced into the analyzer. The entire operation can be performed in 15 to 20 seconds.

Analyses of bubbles taken from the large veins of frogs exercised during decompression show a high N<sub>2</sub> content (average values: 95 per cent N<sub>2</sub>, 3.5 per cent CO<sub>2</sub>, 1 to 2 per cent O<sub>2</sub>). In bubbles from frogs which had been exercised prior to decompression a higher CO<sub>2</sub> content was found (6.5 per cent CO<sub>2</sub>).

TABLE VII

*Analyses of the CO<sub>2</sub> Content of Bubbles from Decompressed Dead Rats and Dead Bullfrogs*

Animal	Time dead before decompression	CO <sub>2</sub> content of bubbles
	<i>hrs.</i>	<i>per cent</i>
Rat	1	60
Rat	1	85, 70
Rat	1	60, 68
Rat	1	80, 73
Frog	48	54
Frog	48	36
Frog	30	26
Frog	30	15
Frog	24	26

All animals decompressed to 50,000 feet.

While these figures show a trend, they should not be taken as indicating the true values for bubbles at their site of origin. The limitations of the method give reason to believe that during the early development of these bubbles the CO<sub>2</sub> tension is much higher than that shown in the analysis. Even if the bubbles originate in a region locally very high in CO<sub>2</sub> (e.g. muscular regions during exercise), equilibration to a lower level undoubtedly occurs rapidly as they move outward into the large vessels, where they are first available for analysis. This was tested by injecting bubbles of 50 per cent CO<sub>2</sub> into the blood stream of a frog at sea level. These were removed at various intervals after their injection and it was found that the bubbles equilibrate with the blood in a few seconds. Since the interval of time between the first visible appearance of a bubble in a vein of a decompressed frog and its introduction into the analyzer is several minutes, much of the CO<sub>2</sub> originally present could diffuse out.

Considering these difficulties, the high values for CO<sub>2</sub> obtained in further



analyses of bubbles from dead rats are of particular interest. Rats were killed by electrocution (110 volts, A.C.), and kept at 37°C. for 10 to 30 minutes after death. After subsequent decompression, bubbles were removed at autopsy by the previously described method and analyzed. The CO<sub>2</sub> content of bubbles formed in dead rats is extremely high, ranging from 60 to 80 per cent (Table VII). In the muscles where the bubbles originate the CO<sub>2</sub> tension is undoubtedly still higher, since there is some loss of CO<sub>2</sub> in transferring the bubble to the analyzer, and also since the bubble, as it moved outward into the large veins, would tend to lose CO<sub>2</sub> to the blood and take up N<sub>2</sub>.

Analyses of bubbles from dead frogs gave essentially the same results as those obtained with rats, although in general the values were somewhat lower. The frogs had been dead from "red leg" for 12 to 48 hours before decompression (to 50,000 feet). The results of the analyses are listed in Table VII, and show that the CO<sub>2</sub> content of bubbles formed in dead frogs is high. Here again the values obtained are probably low, due to operation of the factors mentioned previously.

#### DISCUSSION

In the preceding sections we have approached the relation of CO<sub>2</sub> to bubble formation in a variety of ways. Decompression experiments have shown that bubbles form readily in animals under conditions involving high accumulation of CO<sub>2</sub>, and that certain factors lowering the CO<sub>2</sub> tension in these animals also decrease the tendency for bubble formation. Administration of CO<sub>2</sub> directly to living frogs greatly increases the incidence of bubbles on decompression, and actual analyses of bubbles in living as well as dead animals show that CO<sub>2</sub> is present in appreciable amount. Taken together, these experiments indicate an important rôle for CO<sub>2</sub> in bubble development. From a synthesis of the results we offer the following picture with respect to CO<sub>2</sub> and the initiation and growth of bubbles in the normal, living decompressed animal. As long as such animals remain quiescent, CO<sub>2</sub> probably does not reach a state of supersaturation in the body, and has little if any predisposing influence toward bubble formation. With muscular exercise, however, the CO<sub>2</sub> tension may reach high levels locally in the muscles. At these points the accumulated CO<sub>2</sub> may lead to a marked local supersaturation, which in turn greatly increases the ease with which bubbles may be formed there. It does so by reducing the magnitude of mechanical disturbance necessary for creating bubbles *de novo* at that point. Thus the effects of mechanical agitation are greatly accentuated and facilitated. It should be understood, of course, that N<sub>2</sub><sup>2</sup> is

<sup>2</sup>Oxygen and water vapor would also enter into the bubbles, in proportion to their concentration and at a rate depending also on diffusibility, but the concentration of oxygen is much less than that of nitrogen. Direct analysis of bubbles taken from the veins of decompressed frogs and rats shows the presence of oxygen.

also concerned with the initiation of bubbles, but under these conditions may play only a minor rôle in their origin and early growth. It is possible that bubbles in early stages contain a high proportion of  $\text{CO}_2$  and grow very rapidly in size because of the high concentration of  $\text{CO}_2$  molecules in their immediate neighborhood. As bubbles move out into the larger vessels, they move away from local regions of high  $\text{CO}_2$  and equilibrate rapidly with the relatively low tension of  $\text{CO}_2$  in the larger vessels. This loss of  $\text{CO}_2$  is compensated for by a steady increase in  $\text{N}_2$ , which is responsible for further growth and maintenance of the bubbles.<sup>2</sup> It is conceivable that the composition of the bubble may change from largely  $\text{CO}_2$  at the point of origin to predominantly  $\text{N}_2$  in the larger vessels and heart. We have, therefore, the concept of  $\text{CO}_2$  as a facilitator in bubble formation. It greatly increases the ease with which bubbles may be initiated and may be responsible for their rapid growth in early stages of development. At later stages  $\text{N}_2$  is more directly concerned with their further growth and maintenance.

#### SUMMARY

1. Rats killed in a variety of ways (broken neck, nembutal, anoxia, electrocution) may undergo extensive bubble formation when subsequently decompressed from atmospheric pressure to simulated altitudes of 50,000 feet. On autopsy at sea level, large numbers of bubbles are found throughout the vascular system in the majority of animals. These bubbles appear to originate in small vessels deep within muscular regions, later spreading widely in arterial and venous systems. Dead rabbits and frogs also bubble profusely on decompression.
2. Bubble formation in dead animals is attributed primarily to the accumulation of  $\text{CO}_2$ , derived from residual cellular respiration after death, and from anaerobic glycolysis with attendant decomposition of bicarbonates in blood and tissue fluids. If anaerobic glycolysis is inhibited by using sodium iodoacetate as a lethal agent, bubble formation is greatly reduced or lacking on subsequent decompression.
3. Experiments *in vitro* suggest that high concentrations of  $\text{CO}_2$  favor bubble formation by reducing the degree of mechanical disturbance necessary.
4. Administration of  $\text{CO}_2$  in high concentrations to living frogs lowers the minimum altitude (pressure equivalent) at which bubble formation occurs, with exercise, in untreated animals. Pre-treatment with  $\text{CO}_2$  also reduces the degree of muscular activity necessary for bubbles to form in frogs at higher altitudes.
5. Analyses have been made of the gas content of bubbles taken directly from the large veins of decompressed frogs and rats. In living animals the figures obtained indicate rapid equilibration with gas tensions in the blood. Bubbles taken from decompressed dead rats may contain 60–80 per cent  $\text{CO}_2$ .

6. The bearing of these experiments on the mechanisms of bubble initiation and growth in normal living animals is discussed. Reasons are given for suggesting that CO<sub>2</sub>, due largely to its high dissolved concentration in localized active regions, may be an outstanding factor in the initiation and early growth of bubbles which in later stages are expanded and maintained principally by nitrogen.

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# THE RELATION OF EXERCISE TO BUBBLE FORMATION IN ANIMALS DECOMPRESSED TO SEA LEVEL FROM HIGH BAROMETRIC PRESSURES\*

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Compressed-air illness, often referred to as the "bends" or the "chokes," is a well known clinical entity among divers and others working at high barometric pressures. This condition does not develop during the period of sojourn at increased pressure, but appears on return to atmospheric pressure, if the rate of decompression is excessive. Bert (1878) early showed that animals rapidly decompressed from high pressures to sea level contained bubbles rich in nitrogen in the blood stream, and attributed the symptoms of compressed-air illness to the effects of multiple gaseous emboli. More recently, this interpretation has formed the basis for important research of a practical nature, with emphasis on prevention and treatment of symptoms. Contributions in this direction are especially identified with the efforts of Haldane and coworkers (summarized in Haldane and Priestley, 1935), as well as with those of Behnke and associates (summary, Behnke, 1942). These researches have greatly reduced the danger involved in diving and similar operations and have extended the range of pressures within which work may be carried on.

Recently the problem of decompression sickness has received increased attention in connection with aviation medicine. Symptoms similar to those of compressed-air illness may develop in flyers at high altitudes, and in all probability are associated with bubble formation in blood and tissues (Armstrong, 1939). On the basis of these conclusions we have carried out a series of animal experiments on bubble formation at simulated altitudes. The results of this work are reported separately (Whitaker *et al.* (1945); Harris *et al.* (1945)) and show that muscular activity during decompression is an important causal factor in bubble formation. Additional evidence suggests that this action of exercise at simulated altitudes is mediated through the combined effects of

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mechanical factors<sup>1</sup> and accumulated carbon dioxide, which may attain high local concentrations during muscular activity.

As a result of these findings in animals at simulated altitudes, we were led to investigate the importance of muscular exercise for bubble formation in animals decompressed from high barometric pressures to sea level. On theoretical grounds this is of particular interest since a number of factors affecting bubble formation differ in the two cases. More specifically, one important difference arises from the fact that in animals allowed to equilibrate at high barometric pressures, the total tension of dissolved gases in blood and tissues is much greater than in animals at sea level. Consequently during decompression, the same proportional reduction in pressures (e.g. by one-half) will create a higher supersaturation, expressed in gas tensions or numbers of molecules, in the case of the compressed animals. Secondly, the CO<sub>2</sub> tension of blood and tissues is relatively independent of external changes in barometric pressure. At high barometric pressures, therefore, the CO<sub>2</sub> tension is small relative to the external pressure; at low barometric pressures (simulated altitudes) the CO<sub>2</sub> level is much nearer the total external pressure. The significance of this point lies in the fact that at simulated altitudes, an increase of CO<sub>2</sub> during muscular activity may enable the CO<sub>2</sub> tension to rise locally and exceed the external barometric pressure. Under these circumstances the local CO<sub>2</sub> tension would also exceed the internal pressure of bubbles forming in such loci. This is evident since environmental pressure changes are transmitted throughout the body, except in bone. Thus the internal pressure of bubbles in the blood stream is substantially identical with, and follows changes in the external barometric pressure. It is clear that if the total pressure within a bubble is less than the tension of dissolved CO<sub>2</sub> in its immediate neighborhood, growth could occur from this source alone. Actually, however, since the internal pressure of the bubble represents the sum of the partial pressures of contained gases, and since nitrogen and oxygen would also be present within the bubble, the partial pressure of CO<sub>2</sub> inside the bubble would be appreciably less than the total internal pressure of the bubble. Hence the tendency for CO<sub>2</sub> to enter the bubble would be even greater. It is understood, of course, that nitrogen would further augment bubble growth at simulated altitudes, for similar reasons. Local supersaturations of CO<sub>2</sub> built up in the way described above, are conceived to be responsible for the facilitating action of CO<sub>2</sub>, not only on growth but also in the initiation of bubbles in animals at simulated altitudes (Harris, Berg, Whitaker, Twitty, and Blinks (1945)).

On the other hand, the importance of CO<sub>2</sub> in the development of bubbles

<sup>1</sup>Harvey (unpublished), Blinks (unpublished), and Dean (1944) have developed the rôle of physical factors in bubble formation. They have shown that in fluids supersaturated with dissolved gases, bubbles do not usually arise *de novo* except in the presence of mechanical agitation.

would appear to be much less in animals decompressed from high barometric pressures to sea level. In this case, the external pressure is much higher, even at the end of decompression (760 mm. at atmospheric pressure) and it is unlikely that  $\text{CO}_2$  tensions in the body would ever exceed or reach this level. Considering also that  $\text{CO}_2$  would constitute a relatively small fraction of the total dissolved gases in the blood of compressed animals, it appears improbable that  $\text{CO}_2$  exerts any great influence on bubble formation in decompression to sea level from increased pressure.

Another point of difference between compressed animals and those at simulated altitudes lies in the complicating effects of anoxia, usually present in experiments at low pressures. In animals decompressed in air from high barometric pressures to sea level, anoxia does not enter the picture. The importance of these differences for an understanding of bubble formation in compressed animals will be evident in the following discussion.

*Materials and Methods.*—A small cylindrical steel chamber was used for compression and the animals compressed in air throughout. Pressures were regulated by gauge and a small continuous flow of air was maintained through the chamber during all experiments. Decompression from increased pressures was rapid, the pressure drop to sea level occurring in a few seconds. Muscular activity after decompression was induced by intermittent electrical stimulation (5 to 25 volts, 60 cycle A.C.). In bullfrogs, this was applied by placing the animals on a copper wire grid; with rats, electrodes moistened with saline were attached to the hind limbs. In experiments involving anesthesia, nembutal (for rats) and urethane (for bullfrogs) were used. Anesthetics were administered prior to the compression treatment in all cases where given.

### *Bubble Formation in Compressed Bullfrogs*

*Effect of Exercise.*—Large bullfrogs (*Rana catesbiana*) were compressed in an initial series of experiments to test the effect of exercise, following decompression, on bubble formation. These frogs were all compressed for 1 hour at various selected pressure levels, varying in different experiments from 3 to 60 pounds per square inch.<sup>2</sup> The animals fall into three groups according to the degree of muscular activity on subsequent decompression to sea level. Frogs in the first of these groups were not anesthetized, and were subjected to violent muscular activity immediately after decompression to atmospheric pressure. The exercise, extending over a period of 30 minutes, was violent and maximal, resulting in a state of exhaustion. The animals were then pithed, dissected, and examined carefully for the presence of bubbles under a dissecting binocular microscope.

<sup>2</sup> All pressures given throughout this paper are gauge pressures, in excess of atmospheric pressure. Thus a pressure of 60 pounds per square inch is equivalent to 5 atmospheres absolute pressure.

Animals in the second group likewise were not anesthetized but were not electrically stimulated on return to sea level from increased pressure. Instead, the frogs were allowed to remain quietly in the compression chamber, with slight spontaneous movements, for 30 minutes after reaching sea level. At the end of this time they were pithed and examined as before for bubbles.

TABLE I

*Bubble Formation in Bullfrogs on Decompression to Sea Level from High Barometric Pressures*

Pressure <i>lbs. per sq. in.</i>	No activity (urethanized)	Slight activity (normal, not stimulated)	Violent activity (electrical stimulation)
60	-- --	+++ +-	++ ++
45	-- -	+++ -	++ +
30	-- -	-- --	++ ++
15		--- ---	++ ++
11		--	++ ++
8		-- --	++++ ++++
5		--	++ ++
3		--	++- --

+, bubbles; -, no bubbles. Each symbol represents an individual animal. All frogs compressed for 1 hour.

The third group involved frogs which had been anesthetized with urethane before compression, so that muscular activity was completely lacking, both during the period at increased pressure and on subsequent decompression. After a further interval of 30 minutes at atmospheric pressure, these animals were also dissected and a search made for bubbles.

The results of these three series of experiments are summarized in Table I. Bubbles were present in all frogs of the exercised group which had previously been compressed to 5 pounds or more. In animals pretreated at relatively low pressure levels (5 to 15 pounds), bubbles occurred only in the renal

portal veins and ventral abdominal vein, which drain the hind limbs, region of highest muscular activity during exercise. In frogs that had been at higher pressure levels (30 to 60 pounds) bubbles were present in large numbers in the heart and in all veins and arteries. In the second group of frogs, which were unanesthetized and had not been stimulated, but had slight spontaneous movements, bubbles were present in some cases if the animals had been decompressed from relatively high pressures, but not in frogs treated at 30 pounds or less. In the complete absence of muscular activity, as seen in the group of frogs anesthetized with urethane, bubble formation did not occur in frogs pretreated up to 60 pounds, the highest pressures used in these experiments.

*Minimum Pressure Treatment.*—An additional point of interest concerns the minimum pressure to which bullfrogs must be subjected if bubble formation is to occur when the animals are exercised subsequently at sea level. Two out of five animals pretreated at 3 pounds showed very tiny bubbles in the renal portal veins after prolonged and violent exercise, thus suggesting that the pressure differential of 3 pounds ( $\frac{1}{5}$  atmosphere) may represent the approximate threshold level for bubble formation in compressed bullfrogs. In terms of decompression started at sea level, the reduction of pressure from  $1\frac{1}{2}$  atmospheres to 1 atmosphere is equivalent to a simulated ascent of 5,000 feet. This threshold pressure differential necessary for bubble formation (with exercise) is lower than the comparable threshold altitude (ca. 15,000 feet) in bullfrogs decompressed from sea level (Whitaker, Blinks, Berg, Twitty, and Harris (1945)). That is, a smaller percentage drop in pressure is necessary to cause bubble formation when the decompression starts from higher barometric pressures (above 760 mm.).

#### *Bubble Formation in Compressed Rats*

*Effect of Exercise.*—The results obtained with bullfrogs, as described above, were extended in a further group of experiments with rats. Experimental animals were compressed  $1\frac{1}{2}$  hours at various pressure levels, and following a rapid decompression to sea level were subjected to strong muscular activity for 3 minutes. In a few cases, this was accomplished without direct stimulation merely by inducing the animals to engage in active spontaneous movements. In the majority of instances, however, electrodes were attached to the hind limbs and electrical stimulation applied, resulting in activity of a violent type. The animals were then killed, opened, and the vascular system carefully examined under a dissecting binocular microscope. A parallel control series of rats received identical pressure treatment but were not exercised after decompression. Most of these had been anesthetized with nembutal before compression, to completely inhibit muscular activity; a few animals were unanesthetized but allowed to remain at rest, or with slight voluntary activity,



after decompression. A few of the anesthetized rats were left at atmospheric pressure for 30 to 45 minutes after decompression, but there was no indication that a longer wait before autopsy facilitated the appearance of bubbles in these control animals.

As seen in Table II, bubbles were found in all animals of the exercised group. In rats pretreated at 60 pounds, bubbles were usually present in heart, arteries, and veins; pretreatment at 30 to 45 pounds resulted in bubbles in the veins and right side only of the heart. No extensive study was carried out to determine accurately the lower threshold of pressure necessary for bubble formation in the rat, but each of four rats compressed at 15 pounds after

TABLE II

*Bubble Formation in Rats on Decompression to Sea Level from High Barometric Pressures*

Pressure <i>lbs. per sq. in.</i>	Exercised	Not exercised
60	++ + <sub>1</sub> + <sub>1</sub>	+++ --
45	+++ + <sub>1</sub> + <sub>1</sub>	---- ---
30	+++++ +++++ <sub>1</sub> + <sub>1</sub>	---- ⊖⊖⊖⊖
15	++ ++	-- --

+, bubbles; —, no bubbles; <sub>1</sub>, exercised without electrical stimulation. Exercised rats not anesthetized; all controls (non-exercised group) anesthetized except those circled.

exercise at sea level showed a few small bubbles in the right auricle or post-caval vein. In these rats treated at 15 pounds, therefore, a reduction by half in the external pressure (15 pounds to sea level) was sufficient, with exercise, to cause bubble formation. In terms of decompression started from sea level, this pressure drop is equivalent to a simulated altitude of only 18,000 feet. Previous work by our group has shown that living rats decompressed from sea level do not undergo bubble formation at simulated altitudes of less than 45,000 feet (Whitaker, Blinks, Berg, Twitty, and Harris (1945)). It is evident therefore, that in compressed rats, as in compressed bullfrogs, bubbles will form (with exercise) after a lower percentage drop in pressure than is necessary when these animals are decompressed from sea level to simulated altitudes.

The fact that compressed bullfrogs, under conditions of exercise, will bubble more readily than rats (*i.e.* with a lesser pressure treatment) may possibly be related to the more rapid respiratory turnover and elimination of dissolved

gases after decompression in the rat. A similar difference between the two forms is seen in decompression from sea level to simulated altitudes.

In the control group of non-exercised rats, as shown in Table II, bubbles were not found in any animals pretreated at 15, 30, or 45 pounds. All of these animals were anesthetized except for a group of four rats treated at 30 pounds as indicated by circles in Table II. These unanesthetized but relatively quiet animals likewise failed to show bubbles, indicating that anesthesia is not in itself the explanation for the negative results in control animals. It is clear, therefore, that in rats pretreated within the 15 to 45 pound pressure range, exercise is demonstrably related to the appearance of bubbles, while in the absence of strong muscular activity no bubbles may be seen at autopsy.

*Bubble Formation without Muscular Activity.*—By contrast with the foregoing statement, Table II also shows that in rats pretreated at 60 pounds, bubbles were present in both exercised and non-exercised groups. The occurrence of bubbles, however, was more uniform in the exercised rats given this pressure treatment. In the exercised animals which had been at 60 pounds, bubbles were present in all arteries and veins; frequently the large vessels were completely filled with gas. Three out of five anesthetized rats pretreated at 60 pounds contained large numbers of bubbles at autopsy, although muscular activity was completely lacking throughout the experiments. This finding suggests that bubbles may develop in the complete absence of voluntary muscular activity, if the level of supersaturated gases in blood and tissues is sufficiently high.

E. Newton Harvey and associates (unpublished) have likewise found that in some cases bubbles will occur in compressed cats without muscular exercise (animals given nembutal) if the preliminary pressure treatment exceeds 3 to 3.5 atmospheres absolute (30 to 37.5 pounds gauge pressure). In these anesthetized animals, however, electrical stimulation of the hind limbs greatly increased the rate at which bubbles appeared. The fact that a lesser pressure treatment (30 to 37.5 pounds) is required for bubbles to form without exercise in cats, as compared to rats (60 pounds) correlates with the difference in size, and previous findings that cats bubble more readily than rats at simulated altitudes.

That bullfrogs anesthetized with urethane did not bubble without muscular activity when pretreated at 60 pounds may reflect an incomplete equilibration at the higher pressure, due to the inefficient lung and ventilation mechanism of the frog.

*Effect of Anoxia.*—Rats were also employed to test the possible effect of anoxia on bubble formation in compressed animals. Experimental animals were compressed to 30 pounds for  $1\frac{1}{2}$  hours, then decompressed to sea level, and immediately placed in a closed chamber, to which nitrogen was added slowly at atmospheric pressure. Administration of nitrogen was increased

gradually until, after about 4 minutes, death occurred from anoxia. A moderate amount of muscular activity occurred during this period, particularly just prior to cessation of breathing. No bubbles were observed at autopsy in any of these experimental animals. As a control series, another group of rats was compressed to 30 pounds for  $1\frac{1}{2}$  hours, then decompressed to sea level, and immediately subjected to violent muscular exercise for 3 minutes. Bubbles were found at autopsy in all of these animals (see Table III). Thus, bubbles did not appear in the experimental animals with anoxia and even a moderate amount of exercise, although with a slightly higher level of exercise, bubbles were found in all controls. These results indicate that if anoxia has any facilitating effect on bubble formation in compressed animals, it is too slight to be revealed by the present experiments.

TABLE III  
*Anoxia and Bubble Formation in Compressed Rats*

Treatment after decompression to sea level	Degree of exercise	Autopsy
Killed by anoxia	Moderate (spontaneous)	---
No anoxic treatment (controls)	Violent (stimulated)	+++ +++

+, bubbles; -, no bubbles. All animals compressed at 30 pounds per square inch for  $1\frac{1}{2}$  hours.

#### DISCUSSION

From the data given in the preceding sections, it is clear that muscular activity has a definite predisposing effect toward bubble formation in rats and frogs decompressed from high barometric pressures to sea level, just as previously found in decompression of these animals from sea level to simulated altitudes (Whitaker, Blinks, Berg, Twitty, and Harris (1945)). This finding is particularly interesting in view of the statement commonly made in textbooks that exercise during decompression is beneficial in preventing compressed-air illness in divers and caisson workers. For example, Haldane and Priestley (1935, p. 352) state that "During decompression, or immediately after it, it is desirable that as much muscular work as possible should be carried out, so as to increase the circulation, and therefore the rate of desaturation . . . (in) . . . the body." It may be well to re-examine such conclusions in the light of more extensive information on the effects of exercise. The animal experiments described here suggest that the theoretical benefits of exercise on desaturation are definitely outweighed by a facilitating effect on bubble formation.

The results of exercise as reported here also direct attention to another commonly accepted view, namely that bubbles are not liberated under decompression unless the barometric pressure is reduced to one-half or less of its initial value. For human work under increased pressures, this is expressed by Haldane and Priestly (1935, p. 337) as follows, "...there is a complete immunity from symptoms (in divers) ... if the excess of atmospheric pressure does not exceed  $1\frac{1}{2}$  atmospheres. ... Thus bubbles of nitrogen are not liberated within the body unless the supersaturation corresponds to more than a decompression from a total pressure of  $2\frac{1}{2}$  atmospheres." While the empirical value of this generalization has been proved abundantly for man in practical operations, it is obvious from the present experiments that under conditions of exercise, bubbles may develop in animals at lesser supersaturations. Thus bubbles occurred in exercised bullfrogs following decompression from a total pressure of only  $1\frac{1}{2}$  atmospheres to 1 atmosphere absolute pressure (3 pounds to sea level). Even in rats, where no systematic attempt was made to determine a minimum pressure treatment, bubbles were found on decompression from 2 to 1 atmosphere (15 pounds to sea level), provided the animals were subjected to strong muscular activity.

It seems unlikely that the facilitating effect of muscular activity in the compressed animals is due in any large measure to  $\text{CO}_2$ . As pointed out earlier, under these conditions  $\text{CO}_2$  forms a relatively small fraction of the total dissolved gases in blood and tissues. The high supersaturation of dissolved nitrogen, combined with the mechanical factors involved in muscular exercise (turbulence and similar effects) would appear to be more important here. This does not detract in any way from the important rôle which  $\text{CO}_2$  plays for the development of bubbles in animals decompressed from sea level to simulated altitudes, where supersaturation of this gas may occur in connection with muscular activity.

Mention has been made of the fact that a lesser percentage drop in pressure is required to produce bubbles, with exercise, in animals decompressed to sea level from increased pressures, as compared with those at simulated altitudes. In formulating an explanation, it is significant that the same relative drop in pressure results in a higher supersaturation of dissolved gases in the case of the compressed animals, due to the greater molecular concentration in blood and tissues. That an increased molecular concentration may result in a decrease in the degree of decompression necessary for bubble formation is indicated by the results of treating frogs with  $\text{CO}_2$  and decompressing them to simulated altitudes (Harris, Berg, Whitaker, Twitty, and Blinks (1945)). Bubble formation in these animals was greatly facilitated at all altitudes, and in addition occurred at altitudes well below the minimum, or threshold altitude at which bubbles will form in untreated frogs. In the case of compressed

animals the relatively high level of dissolved  $N_2$  may have a similar action, as expressed in the comparatively low pressure differential necessary for bubble formation. With pressure treatments of greater magnitude, still higher supersaturations of  $N_2$  may reduce the degree of activity required to initiate bubble formation. Thus bubbles occurred in bullfrogs with only slight spontaneous movements after compression at 45 or 60 pounds, although no bubbles appeared under similar conditions of activity if the pressure treatment had been less. Furthermore, bubbles occurred in anesthetized rats pretreated at 60 pounds in the complete absence of voluntary activity. Here presumably the slight mechanical agitation involved in breathing and other vital activities was sufficient to initiate bubble formation. The action of dissolved  $N_2$  in compressed animals, therefore, would appear to be similar in principle to the predisposing influence of  $CO_2$  at simulated altitudes. The effects of the two possibly have a common basis in the facilitation of bubble formation by high molecular concentration in excess of the saturation level.

#### SUMMARY

1. Bullfrogs (*Rana catesbiana*) and rats have been subjected to high barometric pressures and studied for bubble formation on subsequent decompression to sea level. Pressures varying from 3 to 60 pounds per square inch, in excess of atmospheric pressure, were used.

2. Muscular activity after decompression is necessary for bubble formation in bullfrogs after pressure treatment throughout the above range. Anesthetized frogs remained bubble-free following decompression. Rats compressed at 15 to 45 pounds per square inch likewise did not contain bubbles unless exercised on return to sea level.

3. Bubbles form without voluntary muscular activity in anesthetized rats previously subjected to pressure of 60 pounds per square inch. Small movements involved in breathing and other vital activities are believed sufficient to initiate bubbles in the presence of very high supersaturations of  $N_2$ .

4. Bubbles appear (with exercise) in rats previously compressed at 15 pounds per square inch, and in bullfrogs subjected to pressure at levels as low as 3 pounds per square inch above atmospheric pressure. The percentage drop in pressure necessary for bubble formation is less in compressed animals than in those decompressed from sea level to simulated altitudes.

5. The action of exercise on bubble formation in compressed frogs and rats is attributed to mechanical factors associated with muscular activity, combined with the high supersaturation of  $N_2$ .  $CO_2$  probably is not greatly involved, since its concentration does not reach supersaturation, as it does at high altitude.

6. Anoxia following decompression from high barometric pressures has no observable facilitating effect on bubble formation.

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# ADDITIONAL MECHANISMS FOR THE ORIGIN OF BUBBLES IN ANIMALS DECOMPRESSED TO SIMULATED ALTITUDES\*

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In a series of studies, described separately (Whitaker *et al.* (1945), Harris *et al.* (1945)), it has been shown that muscular activity causes bubbles to form in decompressed animals, and that high blood concentrations of dissolved gases (e.g. CO<sub>2</sub> or air) facilitate this effect, decreasing the degree of muscular activity required. Aside from the facilitating effect of the CO<sub>2</sub> produced, the muscular activity is believed to exert its effect largely through the physical consequences of mechanical disturbance. Blinks (unpublished), Dean (1944), and E. Newton Harvey (unpublished) have shown in models that mechanical agitation, by creating "negative pressures," causes bubbles to form in fluids (including blood) that are supersaturated with gases. However, the presence of "nuclei" (e.g. minute bubbles or gas films) which serve as centers for bubble growth is apparently necessary.

Assuming that nuclei are involved in bubble formation in animals, it is important to investigate means by which they may form within the body, or be introduced from without. In the experiments to be described, a possible source of entry of nuclei has been explored and also new methods of producing nuclei and bubbles within the body in the absence of muscular activity have been found.

## *Ingestion of Frothy Fat Emulsions*

Blinks (unpublished) and Dean (1944), have found in models that surfaces of hydrophobic substances such as lanolin, paraffin, etc., in water tend to retain minute air films with great tenacity. If the surrounding water is supersaturated with gases, these air films act as nuclei for the growth of visible bubbles. In addition to retaining air films, hydrophobic surfaces may possibly also be involved in actual *de novo* formation of nuclei (Harvey, unpublished).

These considerations suggested that heavy fat ingestion, especially of colloidal frothy fat with extensive air films, might introduce bubble nuclei into the lymph or blood stream by direct transference through the intestinal mucosa.

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Furthermore, increased fat in the vascular system might favor *de novo* formation of nuclei merely because of the properties of hydrophobic surfaces.

Rats were forcibly fed a large amount (approximately 15 to 20 cc.) of frothy emulsion containing approximately 50 per cent water, 40 per cent beef suet, 10 per cent lanolin, and 0.1 to 1 per cent bile salts. Bile salts were included to facilitate absorption of the fat, and the emulsion was prepared in an electric mixer. At intervals varying from  $4\frac{1}{2}$  to 25 hours after the feeding, the rats were decompressed rapidly in pure  $O_2$  to 50,000 feet,<sup>1</sup> where they were maintained for 2 minutes before recompression and autopsy. For stimulation during decompression, electrodes were attached to the hind limbs.

Eight of these rats engaged in a degree of muscular activity classed as "moderate" which is approximately threshold for bubble formation in normal control rats at this altitude (see also Table II in Whitaker, Blinks, Berg, Twitty, and Harris (1945)). Accordingly, any appreciable facilitating effect on bubble

TABLE I  
*Effect of Fat Ingestion on Bubble Formation in Rats*

Time interval between feeding and decompression	Simulated altitude	Muscular activity	Bubbles present	Bubbles absent
	<i>ft.</i>			
$4\frac{1}{2}$ -25 hrs. ....	50,000	Moderate	0	8
19-20 $\frac{1}{2}$ hrs. ....	50,000	Violent	2	1
Controls. ....	50,000	Moderate	0	3
(See also Table II in Whitaker <i>et al.</i> ) ....	50,000	Violent	5	0

formation resulting from the ingested fat should have been revealed by the formation of bubbles in these animals. Actually, however, no bubbles were found. The muscular activity of three of the experimental rats was violent, and two of these indeed formed bubbles, but this is also characteristic of violently exercised control rats. Therefore, the results (Table I) indicate that ingestion of fat does not favor bubble formation.

Examination at autopsy revealed large quantities of ingested fat in the lymphatic vessels of the experimental rats, especially in the cisterna chyli and mesenteric branches, while these vessels were relatively clear in the controls.

The effect of heavy fat ingestion was further tested in bullfrogs, which form bubbles more readily than rats. Ten animals were each forcibly fed approximately 15 to 20 cc. of an emulsion prepared by blending equal parts of melted nucoa and water, with the further addition of a small amount of NaCl. Eight other frogs were fed a beef suet emulsion, and six were fed an emulsion of beef suet, lanolin, and bile salts. Periods of 2 to 72 hours after feeding were per-

<sup>1</sup> All experimental altitudes referred to in this report are simulated in a decompression chamber.

mitted to elapse, for digestion and absorption, before the frogs were decompressed.

The results are shown in Table II and indicate that the incidence of bubble formation in the experimental frogs with slight muscular activity, which is approximately threshold for bubble formation in normal control frogs at this altitude (see also Table I in Whitaker, Blinks, Berg, Twitty, and Harris (1945)), is not measurably greater than in the controls.

It thus appears that bubble nuclei do not cross the intestinal wall with digested fat in rats and bullfrogs. Furthermore, high fat concentration *per se* in the lymph and blood stream did not facilitate bubble formation. It should be emphasized in passing that the fat in these experiments is concentrated in

TABLE II  
*Effect of Fat Ingestion on Bubble Formation in Bullfrogs*

Time interval between feeding and decompression	Simulated altitude	Type of fat	Muscular activity	Bubbles present	Bubbles absent
	<i>ft.</i>				
2-24 hrs.....	50,000	Nucoa emulsion	Slight	1	6
2-24 hrs.....	50,000	Nucoa emulsion	Moderate	2	1
7-25 hrs.....	50,000	Suet emulsion	Slight	0	6
5-25 hrs.....	50,000	Suet emulsion	Moderate	2	0
28-72 hrs.....	50,000	Suet and lanolin emulsion and bile salts	Slight	2	4
Controls (see also Table I in Whitaker <i>et al.</i> ) .....	50,000	—	Slight	2	6

the lymph and blood, and the results do not bear on the effects of tissue adiposity that might result from a protracted high fat diet.

#### *Crystallization in the Body*

Blinks and Pease (unpublished) have observed in models that crystallization of a number of substances in water, including caprylic acid, induces formation of bubble nuclei *de novo*. Under decompression with mechanical agitation the nuclei grow to form visible bubbles, or in some cases may grow spontaneously without agitation. These results suggested experiments on the rôle of crystallization within the body.

Nuclei-free liquid caprylic acid (melting point 16°C.) was injected into veins of frogs at 25°C. which were then at once cooled at 10°C. The cooling caused caprylic acid crystals to form in the blood stream, and immediately after decompression to 40,000 feet numerous bubbles appeared in the blood vessels

containing the crystals, but not elsewhere. The bubbles grew spontaneously, without mechanical agitation, presumably from centers or nuclei produced by crystallization of the caprylic acid. Controls that were not cooled, in which injected caprylic acid remained liquid, did not form bubbles.

This experiment suggested the possibility that bubble nuclei might arise in normal animals by crystallization of substances occurring naturally in the body. Since cooling might crystallize some unknown organic compound, bullfrogs were cooled in an ice bath until the body temperature dropped to  $1-2^{\circ}\text{C}.$ , after which they were decompressed without exercise to 50,000 feet. The results were negative, in that no bubbles were found on subsequent dissection.

In the course of the experiment just described it was discovered, however, that bubbles appeared spontaneously, without muscular activity, when frogs cooled below  $0^{\circ}\text{C}.$  were subsequently decompressed. Frogs cooled to  $-5^{\circ}$  to  $-8^{\circ}\text{C}.$ , warmed to  $20^{\circ}\text{C}.$  (body temperature), and then decompressed, contained many bubbles in the vascular system. Dissection of frogs immediately after removal from the cold bath disclosed ice crystals in the blood stream.

To test the apparent relation between freezing and formation of bubble nuclei, a bullfrog was anesthetized and wrapped in a towel with one leg extending free. The foot of this leg was placed in a bath of mineral oil at  $-5^{\circ}$  to  $-10^{\circ}\text{C}.$  for nearly an hour. The frog was then dissected to expose the femoral veins, and decompressed to 50,000 feet without muscular activity. The frozen foot was warmed by means of a strong light, to restore circulation, and after a few minutes bubbles appeared in the femoral vein draining blood from the treated foot. This experiment was repeated a number of times and without exception bubbles came from the frozen appendage but not from the other leg. Microscopic examination of frozen feet before decompression revealed that blood in the smaller vessels was frozen solid. In several cases bubbles were observed at sea level (*i.e.* without decompression) in these small vessels after the blood melted.

Small segments of blood-filled veins (ventral abdominal, renal portal) were tied off with thread and removed from the frog. No bubbles formed in any of these segments after decompression. However, when ice crystals were formed in the blood by bringing the segment in contact with dry ice, and the segment was then decompressed, it immediately filled with gas bubbles.

The effectiveness of freezing in forming bubble nuclei was further tested with evacuated test tubes containing frog's blood. Usually no bubbles appeared if the test tube was very clean, indicating that no bubble nuclei were present. However, if the lower portion of the tube was placed in contact with dry ice, a heavy stream of bubbles appeared as soon as ice crystals formed, and it came from the region of the crystals. If the ice crystals were allowed to melt, the heavy stream of bubbles ceased.

It is evident, therefore, that in the animal as *in vitro* crystallization of water forms bubble nuclei and small bubbles. Dissolved gases are forced out of

solution, as in the manufacture of ice. The small bubbles persist in animals for some time after thawing.

### *Fracturing of Bones*

Harvey (unpublished) has found with cats that crushing the leg muscles results in the formation of bubble nuclei. This is confirmed with bullfrogs, but breaking the leg bones is found to be still more effective.

Leg muscles of anesthetized bullfrogs were severely crushed by repeated pounding with a hammer at sea level. The skin was not broken, but hemorrhagic areas were observed in the muscles. The frogs were decompressed to 45,000 feet for 10 minutes, recompressed to sea level, and autopsied. As shown in Table III, bubbles were found in a few of the frogs. Since 45,000 feet is approximately threshold for this effect it is a favorable altitude for comparing the relative effectiveness of bone breaking. When the tibia or femur is broken by hand with a minimum of tissue damage, without breaking the skin, and essentially without injury to the muscles, decompression to 45,000 feet results in the formation of many bubbles in almost all of the frogs. The results are

TABLE III

*Effect of Crushing Muscles and Fracturing Bones on Bubble Formation in Bullfrogs*

Treatment prior to decompression	Simulated altitude	Bubbles present	Bubbles absent
	<i>ft.</i>		
Leg muscles crushed.....	45,000	2	8
Leg bones (femur or tibia) fractured.....	45,000	9	1

shown in Table III, and indicate that bone breaking is considerably more effective than crushing the muscles. If a tibia is broken and the overlying skin is removed, the broken ends of the bone are visible through the intact fascial layers. When such a preparation is decompressed the bubbles can clearly be seen to originate in the region of the bone fracture.

Bubble nuclei are evidently produced by the breaking of the bone. They have been shown to persist at sea level for  $\frac{1}{2}$  to 1 hour, depending in part on the size of the bone, and may be carried to other parts of the body. The mechanism of formation of these nuclei has not been established, but they are probably produced by the intense mechanical disturbances developed when the bone snaps.

### SUMMARY AND CONCLUSIONS

1. A heavy ingestion of frothy emulsified fat by rats and bullfrogs does not increase susceptibility to bubble formation when the animals are decompressed 2 to 72 hours later. This indicates that gaseous films (bubble nuclei) initially present do not pass across the intestinal wall with the digested fat, and also

that high fat content *per se* in the lymph and blood does not increase susceptibility to bubble formation.

2. Liquid caprylic acid injected into veins of bullfrogs crystallizes when the frogs are cooled. The crystallization causes bubbles to form without muscular activity on subsequent decompression. Cooling normal bullfrogs to 1–2°C. fails, however, to crystallize any substances occurring naturally in the animals that might act in a similar manner.

3. When bullfrogs are cooled (*e.g.* to  $-5^{\circ}$  to  $-10^{\circ}\text{C}.$ ) until ice forms in the blood vessels, and are then warmed and decompressed, bubbles form in the absence of exercise. Crystallization of water in the body thus forms nuclei or even small bubbles that persist. If only one foot is frozen, bubbles originate in the frozen foot. In some cases visible bubbles were observed in thawed feet at sea level (*i.e.* without decompression). When frog's blood is partly frozen in test tubes or in tied off sections of veins, bubbles will appear on decompression in the absence of mechanical agitation. The practical relation of this phenomenon to flight at high altitude should not be overlooked.

4. Fracturing a leg bone (tibia or femur) in a frog induces bubble formation on subsequent decompression. Bubble nuclei, which persist for  $\frac{1}{2}$  to 1 hour, are probably formed as a result of the intense mechanical disturbance when the bone snaps. Fracturing of bone is considerably more effective than crushing muscles for producing bubbles in frogs.

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# SOME EFFECTS OF DERIVATIVES OF VITAMIN K ON THE METABOLISM OF UNICELLULAR ALGAE

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Dam, Schoenheyder, Glavind, and others (1) observed that green plants, more specifically the chloroplasts, contain a substance which, if given to animals and men in minute quantities, showed antihemorrhagic properties. Vitamin K<sub>1</sub>, as the substance was called, has been identified as 2-methyl-3-phytyl-1,4-naphthoquinone. The number of publications reporting the effects of vitamin K and its derivatives upon the animal body is rapidly increasing (1). By contrast, the question of the effect of vitamin K<sub>1</sub> on its place of origin (or in bacteria which produce vitamin K<sub>2</sub>) has been neglected.

The present study was undertaken because it lies, obviously enough, in the path of investigations concerning the problem of photosynthesis. It yielded results that can be summarized by saying that there is hardly a metabolic process in algae which cannot be influenced by small amounts of vitamin K.<sup>1</sup>

The substances used in the experiments described below were not genuine vitamin K<sub>1</sub> but derivatives without the phytol side chain, such as (a) 2-methyl-1,4-naphthoquinone, known as synthetic vitamin K or "menadione," (b) 2-hydroxy-3-methyl-1,4-naphthoquinone, known as "phthiocol," (c) several water-soluble naphthoquinone derivatives and compounds in use for clinical purposes. 2-methyl-1,4-naphthoquinone has exactly the same antihemorrhagic potency per mole as the natural vitamin K, the phytol group being of no detectable physiological importance. In the following we shall refer to the 2-methyl-1,4-naphthoquinone as vitamin K, to the substance containing an additional hydroxyl group as phthiocol.

The methods employed to measure the gas exchange—being manometric according to Warburg—need no comment. The cultivation of the species of algae investigated—namely *Chlorella pyrenoidosa*, *Scenedesmus obliquus*, *Scenedesmus* sp. D<sub>8</sub>—have been described often before.

The naphthoquinones were added as buffered aqueous solution of known concentration, whenever the solubilities of the substances in question permitted. Phthiocol forms a Bordeaux red colored solution in alkaline phosphate. The color disappears upon acidification or reversible reduction of the quinone with hydrosulfite. The

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<sup>1</sup> Independently Dam has found that crystals of 2-methyl-1,4-naphthoquinone, if left in contact with a suspension of algae, cause an inhibition of photosynthesis (*Am. J. Bot.*, 1944, **31**, 492).

commercial water-soluble antihemorrhagic drugs were dissolved and filtered shortly before use, because the solutions had a tendency to become turbid on standing. On account of its poor solubility vitamin K was used in reproducible but undetermined concentrations. Either the dry crystals were added in excess to the medium containing the plants or a saturated clear solution from freshly precipitated material was prepared which could be diluted in desired proportions.

The tables present rates of gas exchange measured in vessels of identical volume giving directly comparable readings. In cases where the type of gas exchange could be determined the metabolic quotients were calculated from the cubic millimeters of gas exchanged per milligram of dry weight of cells.

### Respiration

Small concentrations of the naphthoquinones stimulated the respiration in all algae. The extent of the stimulation varied with the drug and with the species. The strongest stimulating effects were those of phthiocol upon the respiration in *Chlorella pyrenoidosa*.

The degree of stimulation depends on the respiratory rate of the untreated control. In starved *Chlorella* this rate can be rather small (e.g.  $Q_{O_2} = 0.3$ ). By adding  $10^{-4}$  M phthiocol to such low respiring cells the rate of respiration may be raised fifteen times. The absolutely highest rates were found, however, with cells having a normal  $Q_{O_2}$  (= rate: c.mm./mg. dry weight/hour) between two and five. The maximum stimulation remains below that obtainable by adding glucose to a suspension of normal *Chlorella* cells (cf. 2). If the respiration of such cells has been stimulated before with phthiocol addition of glucose does not bring the rate of respiration to the level of the normal cells fed with glucose. By comparison, the effect of phthiocol appears now as an inhibition (Tables I and II). This behavior reminds one of the observations made by Emerson (2) and Genevois (3) that in *Chlorella* cyanide stimulates the oxidation of internal material but inhibits that of substances entering the cell from outside. The effect of the methyl-naphthoquinones is clearly catalytic. Much more oxygen is taken up under the influence of phthiocol or vitamin K than can be accounted for by their oxidation.

Since larger concentrations of the naphthoquinones inhibit respiration there exists an optimum of the stimulated rate. The effect, therefore, resembles the action of dinitrophenols upon respiring cells in general (4, 5), including algae (6, 7). If crystals of vitamin K are added to the algal suspension to demonstrate the stimulating effect, the stimulation changes slowly into an inhibition which eventually becomes complete (Table III). Using a 1/40th saturated solution of vitamin K the stimulation can be shown to persist for a long time. The absolute rate of the stimulated respiration depends, on the other hand, on the combustible material available. Table IV shows that both the normal and the stimulated respiration in *Chlorella* become smaller with time but regain their original value after a period of illumination (that is, of photosynthesis).

TABLE I

*Stimulation of Respiration in Chlorella pyrenoidosa by Phthiocol*

Summary of several experiments. Cells suspended in phosphate solutions varying in pH from 4.9 to 6.2. Temperature 25°. Gas phase: air, in absence or presence of 6 per cent KOH. Rates of oxygen consumption given as  $Q_{O_2}$  = c.mm./mg. dry weight/hour. Respiratory quotients (R./Q.) refer to periods averaging 1 hour. (a) and (b) fresh cultures, (c) old and starved culture.

Time elapsed after start of experiment	Control		With $1 \times 10^{-4} M$ phthiocol	
	$Q_{O_2}$	R.Q.	$Q_{O_2}$	R.Q.
(a) 1	4.2	0.93	8.2	1.1
2	2.4	1.10	7.0	0.95
6	2.0	1.10	4.0	0.95
Now with 0.5 per cent glucose				
8	20.0	1.00	5.0	0.93
(b) 1	5.5	0.98	12.5	1.10
Now with 0.5 per cent glucose				
2	12.0	0.98	10.0	1.01
(c) 2	0.5	2.00	6.8	0.95
9	0.3	1.55	3.8	0.82
20	0.2	—	1.7	—
Now with 0.5 per cent glucose				
23	7.5	—	2.1	—
25	7.5	1.25	5.1	0.85

TABLE II

*Influence of Phthiocol (2-Methyl-3-Hydroxy-1,4-Naphthoquinone) upon Respiration and Compensation in Chlorella pyrenoidosa*

0.067 cc. of cells in 4 cc. of  $M/30$  phosphate buffer pH 6.2. Temperature 25°. Gas phase: air in presence of 6 per cent KOH on filter paper.

Concentration of phthiocol .....	0.0	$10^{-5} M$	$10^{-4} M$	$5 \times 10^{-4} M$
Rates of oxygen exchange, c.mm./10 min.				
(a) Dark. Respiration .....	-2.5	-5.0	-12	-17
(b) Light. 4,000 lux. Compensation corrected for respiration .....	+4.1	+7.7	+12.7	+10.5
Glucose added to all samples; concentration 0.5 per cent				
(c) Dark. Respiration with glucose .....	-31	-27	-21	-10
(d) Light. Compensation with glucose corrected for respiration .....	+43	+39	-5	$\pm 0^*$

\* A compensation rate of zero means that the absorption of oxygen continues in the light at the same rate as before or afterwards in the dark. A compensation rate equal to that of respiration means that in the light no measurable exchange of oxygen can be observed.



TABLE III

*Respiration of Chlorella pyrenoidosa in Presence of Crystals of Methyl-1,4-Naphthoquinone (Vitamin K)*

Conditions like those given in Table I. R.Q. rose from 0.76 to 0.90 in the poisoned algae during the experiment as compared with 0.93 to 1.10 in the normal cells.

Time elapsed, hrs.....	1	2	6
QO <sub>2</sub> : normal .....	4.2	2.4	2.0
QO <sub>2</sub> : in presence of floating crystals of vitamin K ..	9.7	2.3	0.5

TABLE IV

*Acceleration of Respiration and Inhibition of Photosynthesis in Chlorella pyrenoidosa by 2-Methyl-1,4-Naphthoquinone (Vitamin K) and 2-Methyl-3-Oxy-1,4-Naphthoquinone (Phthiocol)*

0.05 cc. of cells in M/20 phosphate buffer at pH 5.9. Temperature 25°. Gas phase: air, later air with 4 per cent CO<sub>2</sub>. Vessels used in sets of two, with and without 6 per cent KOH on filter paper. During this experiment the respiratory quotients were found to range from 0.80 to 0.95 without much difference between normal and poisoned algae. A solution of vitamin K was prepared by saturating some phosphate buffer with freshly precipitated methyl-naphthoquinone. For the experiment the saturated solution was diluted forty times.

Concentration of poisons .....	Vitamin K		Phthiocol	
	—	1/40 saturated solution	—	1 × 10 <sup>-4</sup> M
Time elapsed after adding the poisons	Rates of absorption of oxygen in QO <sub>2</sub> , (c.mm./mg. dry weight/hr.)			
min.				
20	-2.4	-7.2	-1.3	-5.0
180	-2.4	-3.6	-1.2	-3.9
800	-1.2	-2.0	—	—
	Illuminated with 10 <sup>8</sup> lux during 50 min. in presence of CO <sub>2</sub> . CO <sub>2</sub> removed again.		Washed twice on the centrifuge	
900	-2.7	-6.6	-1.0	-1.1
960	-2.7	-3.0	—	—
	Rates of pressure changes due to photosynthesis at 1,000 lux, mm./10 min.			
800	+24	+25	+18	+3.7
900			+17	+12.5

Though the antihemorrhagic potency of phthiocol is only about one-hundredth of that of vitamin K, both substances have the same effect on the metabolism of algae. Three other substances, "menadione bisulfite," 2-methyl-1,4-naphthoquinone-diphosphoric acid tetra sodium salt, acetoxy-2-methyl-4-

naphthyl sodium phosphate, appear to have an accelerating effect only upon respiration. The data of Table V show that the concentrations of these easily water-soluble substances have to be higher than those of phthiocol for a measurable similar stimulation. A concentration of  $M/2000$  phthiocol already

TABLE V

*Comparison of the Effects of Water-Soluble Derivatives of Vitamin K on Photosynthesis and Respiration in Scenedesmus obliquus*

Concentrations of added substances  $M/2000$ . Numbers represent relative rates of gas exchange, the rates in the normal cells being put at 100.

Time after adding the substances	Phthiocol	Menadione bisulfite*	2-Methyl-1,4-naphthohydroquinone-diphosphoric acid (Na salt)†	1-Acetoxy-2-methyl-4-naphthyl sodium phosphate
<i>hrs.</i>				
	(a) Photosynthesis at approximately 3,000 lux			
3	8	106	100	115
14	0	100	100	—
	(b) Respiration			
8	42	120	150	200
14	60	90	100	—

\* Bisulfite compound of 2-methyl-1,4-naphthoquinone, courtesy of Dr. R. Menotti.

† Courtesy of Dr. H. Dam.

TABLE VI

*Effect of Phthiocol on Respiration in Scenedesmus obliquus and Scenedesmus sp. D<sub>3</sub>*

0.035 cc. of cells in  $M/20$  phosphate buffer at pH 5.0, 6.5, and 7.5. Gas phase: air in presence of 6 per cent KOH on filter paper. To eliminate internal carbon dioxide suspension illuminated during 15 minutes before the measurements.

pH.....	<i>Scenedesmus obliquus</i>			<i>Scenedesmus sp. D<sub>3</sub></i>		
	5.0	6.5	7.5	5.0	6.5	7.5
	$QO_2$ : = rate of respiration, c.mm. $O_2$ /mg. dry weight /hr.					
Normal.....	-0.5	-0.5	-0.5	-1.7	-1.8	-1.3
1 hr. after adding phthiocol						
$4 \times 10^{-5} M$ .....	-2.2	-2.0	-0.7	-2.2	-4.1	-1.3

inhibits the respiration of *Scenedesmus obliquus*, an alga with a type of respiration intermediate between that of *Chlorella* and that of *Scenedesmus D<sub>3</sub>*. It may be that the dissociated sodium salts do not penetrate deeper into the cell and affect only the respiration located in the outer layer. Phthiocol becomes the less effective the lower the concentration of hydrogen ions—the more it exists in the form of the dissociated salt (Tables VI and VIII). Again this reminds one of the well studied action of the dinitrophenols.

### *Photosynthesis*

Vitamin K and phthiocol (but not the other water-soluble derivatives) act as very powerful inhibitors of photosynthesis (Table IV, V, VII, VIII). With very small doses it is possible to stimulate respiration without yet appreciably inhibiting the course of photosynthesis. With increasingly larger concentrations photosynthesis reveals itself as by far the more sensitive process of the two. It stops completely long before the stimulated respiration, after passing the optimum, falls below the original rate in the unpoisoned algae. This and the fact that the inhibition of photosynthesis persists even at very low light intensities classifies the methyl-naphthoquinones as poisons in line with hydroxylamine and *o*-phenanthroline (7). In principle the inhibition is reversible but the lipoid-soluble naphthoquinones are not as easily removed from the cells by washing as cyanide or hydroxylamine. In addition all naphthoquinones if used in higher concentrations damage the cell progressively, particularly under anaerobic conditions. The cells appear slowly to be bleached. Whether light has an accelerating effect on this destruction of chlorophyll has not been investigated.

### *Compensation of Respiration in the Light*

When illuminated, plants convert the carbon dioxide produced in the dark by respiration quantitatively into oxygen. In the absence of any external source of carbon dioxide one would expect the illuminated algae to give off at least some carbon dioxide, but usually they produce a little more oxygen in the light than they absorb. This remains true even if the respiration is stimulated artificially by poisons or added substrates (Tables II and VII). The fact that no carbon dioxide seems to escape from the cell is puzzling. Warburg offered a plausible explanation, namely that the mechanism of photosynthesis catches a precursor of carbon dioxide before the latter is set free in respiration (8). The strange efficiency of the compensation reaction remains, however, the only case—if we accept Warburg's hypothesis—pointing to a linkage between photosynthesis and respiration. Otherwise all measurements of photosynthesis in presence of free carbon dioxide give the most consistent results, if the normal respiration is treated as a completely independent reaction, influenced only indirectly by the supply of finished photosynthetic products. (Compare the discussions in references 6, 9–11.) Certainly, studying the compensation of respiration in absence of carbon dioxide is just as much a method to separate oxygen evolution from carbon dioxide absorption as the technique introduced lately by Umbreit and coworkers (11) who feed benzaldehyde to their cells, thereby obtaining an additional amount of oxygen.

Whatever the nature of the compensation reaction may be, we wish to point out here that the influence of phthiocol on it parallels that on photosynthesis. The proportion of oxygen evolved during compensation to that absorbed in the

dark invariably becomes smaller in the presence of phthiocol. The absolute rate of the compensation reaction, however, first rises with the stimulated respiration. The decline of the rate of respiration and compensation produced by more phthiocol is much steeper for the latter. The evolution of oxygen in

TABLE VII

*Acceleration of Respiration and Inhibition of Photosynthesis by Phthiocol in Scenedesmus sp. D.*

m/30 phosphate buffer pH 6.0. Temperature 25°. Gas phase: air in presence of KOH, later air with 4 per cent CO<sub>2</sub>. Illumination: 4,000 lux.

Concentration of phthiocol.....	0.0	$2 \times 10^{-5} \text{ M}$	$1 \times 10^{-4} \text{ M}$	$0.5 \times 10^{-3} \text{ M}$
Rates of gas exchange, mm./10 min.				
(a) Dark. Respiration in air in presence of KOH.....	-3	-10	-5	-3
(b) Compensation. Effect of light in absence of CO <sub>2</sub> . Corrected for respiration.....	+5.5	+11.5	-1.0	-1.5
(c) Photosynthesis with added CO <sub>2</sub> . Corrected for respiration.....	+37	+30	+3.0	+1.0

TABLE VIII

*Effects of Phthiocol on Photosynthesis, Adaptation, and Photoreduction*

0.03 cc. of cells of *Scenedesmus* in 3.5 cc. of m/25 phosphate solution. Temperature: 25°. Gas phase: air with 4 per cent CO<sub>2</sub>; later H<sub>2</sub> with 4 per cent CO<sub>2</sub>.

pH.....	5.0	6.5	7.3
Rate of pressure changes, mm./min.			
(a) Photosynthesis at 3,000 lux.....	+0.5	+0.67	+1.1*
Same, 5 min. after adding phthiocol, m/10,000.....	±0.0	-0.08	±0.0
(b) Photoreduction after 15 hrs. in H <sub>2</sub>			
700 lux.....	-0.1	-0.5	-0.5
1,300 lux.....	±0.0	-1.0	-1.1
3,000 lux.....	-0.2	-2.0	-2.0
(c) Again photosynthesis			
3,000 lux.....	±0.0	+0.08	+0.15*

\* The increase in rate of pressure changes with pH is apparent only and due to retention of carbon dioxide in the phosphate buffer.

the light may have ceased completely when the respiration has come down only to its original level (Table VII). According to the data contained in Table II the compensation reaction seems to be more sensitive to phthiocol in presence of glucose. With  $10^{-4}$  M phthiocol respiration is accelerated 480 per cent. Light still compensates this high rate completely with an excess of 5 per cent

of oxygen developed over that absorbed in the dark. After adding glucose the respiration in the poisoned algae rises another 75 per cent—but now the illumination is unable to diminish the uptake of oxygen. This observation with phthiocol in the presence of glucose deserves further study.

### *Adaptation and Photoreduction with Hydrogen*

We have said that the methyl-naphthoquinones act upon photosynthesis similarly to hydroxylamine and *o*-phenanthroline. This similarity is confirmed by the observation that phthiocol, etc., inhibit the adaptation to the hydrogen metabolism in *Scenedesmus* (Table VIII). Once the adaptation has taken

TABLE IX

#### *Effect of Phthiocol upon the Oxyhydrogen Reaction*

(a) *Scenedesmus* in phosphate buffer at pH 6.2. Temperature 25°. Adaptation time: 24 hrs. Duration of experiment: 8 hrs.

Concentration of phthiocol.....	—	$0.8 \times 10^{-4} \text{ M}$	$8.0 \times 10^{-4} \text{ M}$	$25 \times 10^{-4} \text{ M}$
Total amount of gas absorbed after addition of 50 c.mm. of $\text{O}_2$ .....	228	202	112	101

(b) 50 c.mm. of oxygen added to adapted algae in hydrogen with 4 per cent carbon dioxide. 0.027 cc. of cells in (1) M/100 and (2) M/25 sodium bicarbonate.

Concentration of phthiocol.....	1		2	
	M/10,000	M/3,000	M/10,000	M/3,000
Time to completion of reaction, min.....	265	215	350	180
Total amount of gas absorbed, c.mm.....	148	147	130	143

place the same substances may serve to stabilize photoreduction at a rate of one-half of the normal one. The problem of stabilization is discussed more fully in the following paper.

### *Coupled Reduction of Carbon Dioxide in the Dark*

Among the complex set of reactions following the absorption of oxygen by algae adapted to hydrogen the coupled reduction of carbon dioxide is the first to disappear under the influence of the methyl-naphthoquinones (Table IX). What is left is either a complete oxyhydrogen reaction or an incomplete one as in the cases already described (12). Under anaerobic conditions phthiocol is reduced by the cells to a colorless form (hydroquinone). The latter is reoxidized by traces of molecular oxygen. Hence it can act as a catalyst for the oxidation of hydrogen in addition to the natural systems in the algae. This might be the reason why the curves representing the course of the oxyhydrogen reaction in

the presence of phthiocol (Fig. 1) deviate somewhat from those already published.

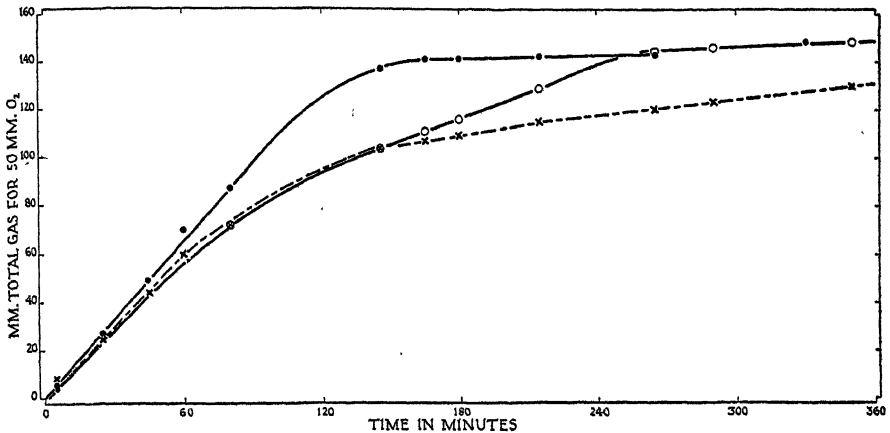


FIG. 1. Oxyhydrogen reaction in presence of phthiocol. 50 mm. (Brodie)  $O_2$  added to adapted *Scenedesmus* in hydrogen with 4 per cent carbon dioxide.

- — ● suspended in M/25 bicarbonate with  $4 \times 10^{-4}$  M phthiocol.
- + — + suspended in M/25 bicarbonate with  $1.3 \times 10^{-4}$  M phthiocol.
- — ○ suspended in M/100 bicarbonate with  $1.3 \times 10^{-4}$  M phthiocol.

#### CONCLUSION

It is remarkable that a substance possessing such powerful catalytic as well as anticatalytic properties is found in the very place where the sensitive enzymatic process occurs, namely in the chloroplasts. Since the vitamin K derivatives tested have the same effect upon photosynthesis and photoreduction as hydroxylamine and *o*-phenanthroline, we can hardly avoid the conclusion that they interact with the photochemical process by forming compounds with one or more heavy metal catalysts. On the other hand, their stimulating influence on respiration and their capacity to transfer hydrogen to oxygen differ from those of the other substances mentioned. In the latter cases, the 1,4-naphthoquinones appear to act as oxido-reduction catalysts. Hence vitamin K (and other naphthoquinones) may serve in more than one way in the metabolism of a plant.

#### SUMMARY

Vitamin  $K_1$ , 2-methyl-3-phytyl-1,4-naphthoquinone, is a substance found in all plant chloroplasts. It is, therefore, interesting to know whether it has any influence upon the metabolism of plants. Experiments made with the phytol-free derivatives like 2-methyl-1,4-naphthoquinone or the corresponding 3-oxy

compound, phthiocol, gave the following results. These substances accelerate the respiration of *Chlorella* or *Scenedesmus* in a way similar to the action of the dinitrophenols. They inhibit photosynthesis and the compensation of respiration in the light strongly like hydroxylamine. In *Scenedesmus* they hinder the adaptation to the anaerobic utilization of hydrogen. If given after adaptation in amounts sufficient to stop photosynthesis they do not prevent photoreduction but rather stabilize this reaction against reversion. Their presence destroys the coupling between the reduction of carbon dioxide in the dark and the oxyhydrogen reaction in adapted algae. One can expect, therefore, that the natural vitamin K present in plants in concentrations of about  $10^{-3}$  M takes part in some metabolic reaction as a catalyst or regulator.

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# *o*-PHENANTHROLINE AND DERIVATIVES OF VITAMIN K AS STABILIZERS OF PHOTOREDUCTION IN SCENEDESMUS

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Among the recently observed effects of specific inhibitors on the metabolism of algae like *Scenedesmus* (1) two are of particular interest because they deviate from the type of inhibitions most frequently described in the literature on cell metabolism. The first concerns the extent of the inhibition and the quantum yield in photoreduction. Poisoning of the algae with hydroxylamine after their adaptation to hydrogen inhibits the rate of photoreduction even at very low light intensities. This means that despite the otherwise favorable conditions the quantum yield of the photochemical process decreases. As expected, the inhibition becomes rapidly greater with increasing concentrations of hydroxylamine. Until now similar effects upon the photochemical process proper have been classified as narcotic and unspecific, since they were found to be characteristic for those obtained with surface-active substances like urethanes, chloretone, chloroform. It is well known that with sufficiently high concentrations of such substances photosynthesis can be stopped completely. Here, however, we face a new problem because no matter how high the concentration of hydroxylamine is made (up to reasonable limits) the rate of photoreduction at low intensities does not drop below 50 per cent of that of the control.

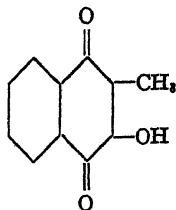
The other observation concerns the reversion (2) (called 'turn back' in the first papers (1)) to the production of oxygen. Adapted cells thoroughly poisoned with hydroxylamine continue to utilize hydrogen for hours at light intensities which cause unpoisoned cells to revert to photosynthesis in a few minutes. The maximum rate of photoreduction thus stabilized by hydroxylamine far surpasses the maximum continuous rate obtainable with unpoisoned cells. Since the concentrations of hydroxylamine used in these experiments were rather high ( $10^{-2}$  M), one might ask whether the peculiar results were caused by reactions different in nature from those generally held responsible for the hydroxylamine poisoning, for instance by the formation of oximes with intermediates.

To decide the question a search was made among substances with anticatalytic properties. This paper presents data showing that 1, 10 (= ortho) phenanthroline





or 2-methyl-3-hydroxy-1,4-naphthoquinone (phthiocol)



can replace hydroxylamine,  $\text{H}_2\text{NOH}$ , in the following cases: inhibition of photosynthesis, of adaptation, of photoreduction, and of the reversion reaction. The three substances causing nearly identical effects seem to have nothing in common chemically but the capacity to form complexes with heavy metal catalysts. The poisoning with high concentrations of hydroxylamine, therefore, does not require any other explanation than that accepted for its activity at small concentrations, namely the inhibition of an enzyme containing iron (or, unlikely, copper). For comparison a few experiments with iodoacetamide and chloretonone are included in this paper.

The atypical inhibition of photoreduction to the limit of only one-half of its normal rate and the simultaneous stabilization against the reversion by strong light point to a particular sequence of reactions in the photosynthetic system. The interpretation of the earlier results have been discussed at length in a recent review (2). The new experiments fit into the detailed diagram of photosynthesis presented there. To be fully understood they require, however, a further theoretical refinement.

Of more general interest to plant physiologists may be the finding that derivatives of vitamin K, which occurs in all green cells, have a widespread influence on the metabolism of these algae. (See the preceding paper.)

### 1. *o*-Phenanthroline

The material (*Scenedesmus* sp.  $\text{D}_3$  and *obliquus*) and the method (manometric) used are identical with those of the earlier publications and need no description here. Fig. 1 shows the influence of increasing concentrations of *o*-phenanthroline upon the rate of photosynthesis and of photoreduction at different light intensities. The rates are given in per cent of the normal rate at 3,000 lux. On account of the quick reversion the "normal" rate of photoreduction at this light intensity (produced and measured with an ordinary incandescent lamp) cannot be observed directly like the rate at 600 lux. The 100 mark on the ordinate of Fig. 1 was found by extrapolating the maximum rate preceding the reversion in experiments of only a few minutes' duration with slightly poisoned algae.

Up to  $0.5 \times 10^{-3} \text{ M}$  *o*-phenanthroline the inhibition effects increase rapidly in all cases shown. Above that concentration the changes are

small. Photosynthesis has practically stopped, whereas photoreduction continues at half the rate of the control. A further increase in the con-

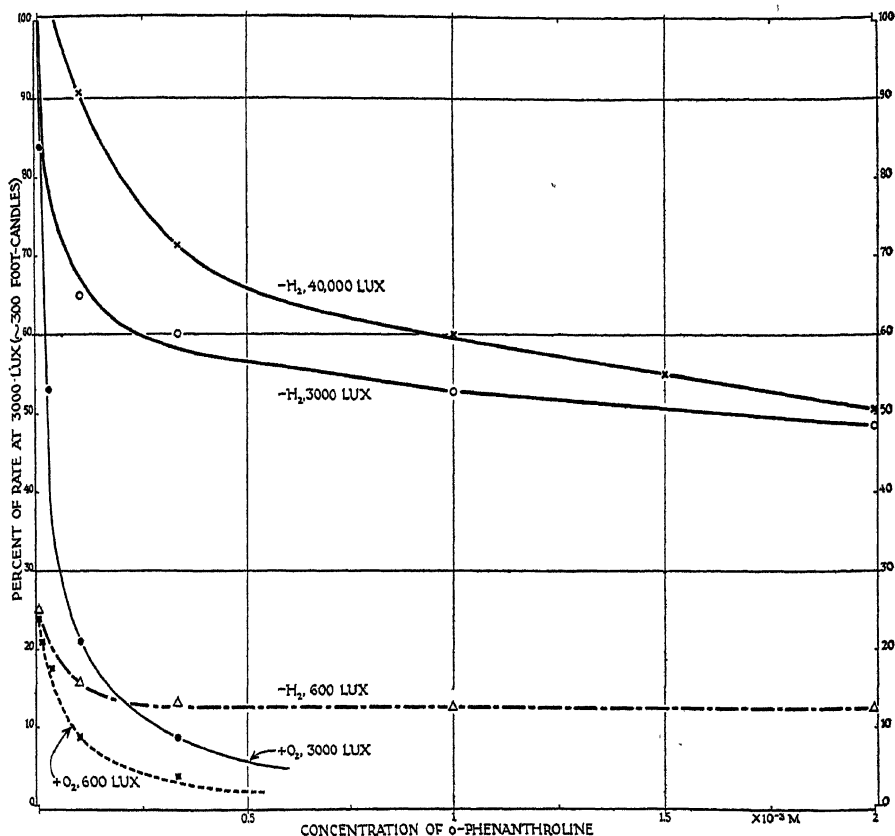


FIG. 1. Inhibition of photosynthesis (+O<sub>2</sub>) and of photoreduction (-H<sub>2</sub>) in *Scenedesmus* by increasing concentrations of *o*-phenanthroline. Rates in percentages of the rate in unpoisoned algae at 3,000 lux (ca. 300 foot-candles). 0.02 cc. of wet cells (4 mg. dry weight) in 3 cc. of M/30 phosphate buffer pH 6.0. Temperature: 26°. Gas phase: 4 per cent of carbon dioxide in air or in hydrogen. Photosynthesis at 600 lux, × --- ×; at 3,000 lux, ● — ●. Photoreduction at 600 lux, Δ --- Δ; at 3,000 lux, ○ — ○; at 40,000 lux, + — +.

centration of the poison has a slight effect at high intensities and none at low ones. Obviously photoreduction makes use of two pathways, one which is easily inhibited by phenanthroline and another which is insensitive. The latter is able to follow a rise of the light intensity in a typical light saturation curve, which is plotted in Fig. 2. Complete protection against reversion at saturation

intensities exists only above an *o*-phenanthroline concentration of  $10^{-3}$  M. With  $10^{-4}$  M phenanthroline reversion occurs at 40,000 lux after 5 minutes. Compared with hydroxylamine phenanthroline is definitely more effective in inhibiting the reversion. We also find that less phenanthroline than hy-

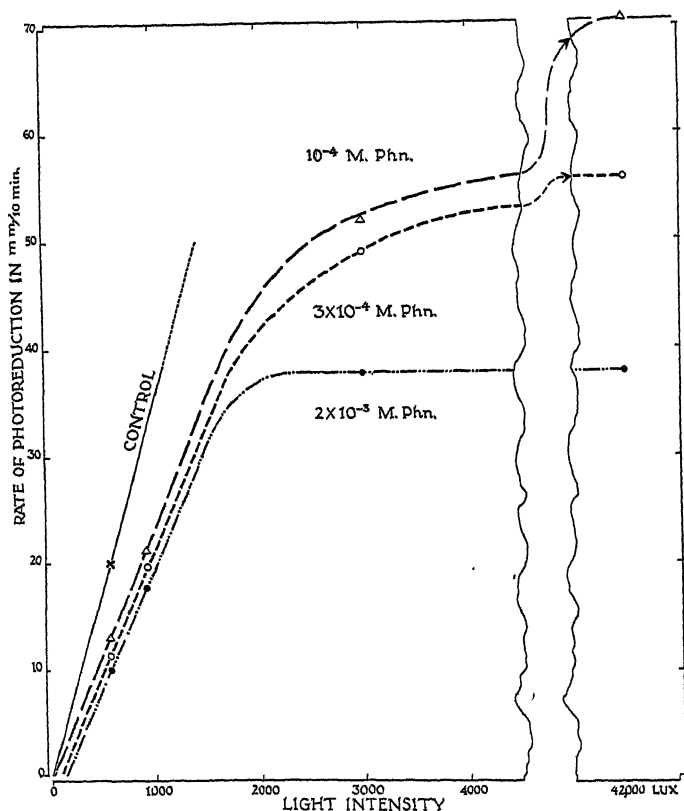


FIG. 2. Light saturation curves for photoreduction of *Scenedesmus* in presence of *o*-phenanthroline.  $\times$  —  $\times$  without *o*-phenanthroline, reverts to photosynthesis at about 800 lux.  $\triangle$  —  $\triangle$  with  $10^{-4}$  M *o*-phenanthroline, saturated at about 20,000 lux, reverts to photosynthesis after 5 minutes at 40,000 lux.  $\circ$  —  $\circ$  with  $3 \times 10^{-4}$  M *o*-phenanthroline.  $\bullet$  —  $\bullet$  with  $2 \times 10^{-3}$  M *o*-phenanthroline.

droxylamine is needed for an inhibition of the adaptation reaction. Table I shows that  $3.3 \times 10^{-5}$  M phenanthroline is sufficient to prevent it, yet this concentration allows the photochemical production of oxygen to proceed at a good, though diminished rate. The effect of phenanthroline upon adaptation hence resembles more that of cyanide than that of hydroxylamine. This is true also for the inhibition of respiration, which in most algae is insensitive to

hydroxylamine. In Table I we see that respiration and photosynthesis in *Scenedesmus* D<sub>8</sub> are inhibited approximately to the same extent by *o*-phenanthroline.

TABLE I

*Inhibition of Adaptation in Scenedesmus by o-Phenanthroline*

0.028 cc. of cells in 4 cc. of M/30 KH<sub>2</sub>PO<sub>4</sub>. Gas phase: air with carbon dioxide, later hydrogen with carbon dioxide. Temperature 26°. Adaptation period: 12 hours.

Concentration of <i>o</i> -phenanthroline.....	0.0	$1 \times 10^{-3} \text{ M}$	$3.3 \times 10^{-3} \text{ M}$	$1 \times 10^{-2} \text{ M}$	$3.3 \times 10^{-2} \text{ M}$
Reaction rates in pressure changes, 10 mm./min.					
Photosynthesis (corrected for respiration)					
3,000 lux.....	+14.8	+12.5	+7.9	+3.1	+1.3
560 lux.....	+3.5	+3.1	+2.8	+1.3	+0.6
Photoreduction after 12 hrs. adaptation and 40 min. illumination					
560 lux.....	-19	-23	$\pm 0$	$\pm 0$	$\pm 0$
Photosynthesis after reversion caused by 3,000 lux.....	+10	+10	+8	+6	$\pm 0$
Respiration in air after elimination of CO <sub>2</sub> by KOH.....	-2.0	-1.9	-1.6	-0.8	-0.3
Respiratory quotients					
R.Q.....	0.96	0.92	1.03	0.94	0.71

TABLE II

*Reversion in Presence of o-Phenanthroline and Cyanide*

*Scenedesmus* D<sub>8</sub> in M/20 bicarbonate. Gas phase: H<sub>2</sub> with 5 per cent CO<sub>2</sub>. Adaptation period 13 hours. Negative numbers mean rates of pressure changes due to photoreduction. Positive numbers mean rates of pressure changes due to photosynthesis after reversion.

	Rates, mm./10 min.			
Without poisons				
560 lux.....	-21	-21	-20	-18
Poisons added.....	-	M/3,000 HCN	M/3,000 <i>o</i> -phenanthroline	M/3,000 HCN + M/3,000 <i>o</i> -phenanthroline
560 lux. (after 40 min.).....	-22	+2	-11	-7
900 lux.....	+6	+4	-18	$\pm 0$

throline. In respect to photoreduction, however, cyanide and phenanthroline behave as antagonists. Table II shows an experiment yielding results similar to those obtained in presence of hydroxylamine and cyanide (compare Fig. 7, reference 1).

The influence of *o*-phenanthroline in concentrations of about  $0.33 \times 10^{-3}$  M upon the dark reduction of carbon dioxide coupled with the oxyhydrogen reaction consists mainly in an inhibition of the coupled reduction, while the oxyhydrogen reaction is allowed to go to completion. In all the experiments described above it is important that the measurements are made within a few hours after adding the poison to the cell suspension. If they last longer than 10 hours the liquid of the cell suspension containing *o*-phenanthroline begins to show a reddish color characteristic for the phenanthroline ferrous iron complex. Simultaneously the inhibitions observed increase in an unpredictable manner and are no more exactly reproducible. Perhaps divalent iron is extracted from the cell which might serve normally in metabolic reactions.

## 2. Phthiocol and Other Vitamin K Derivatives

Vitamin K, phytyl-methyl-naphthoquinone, seems to occur in all plant chloroplasts. It is insoluble in water. 2-methyl-1,4-naphthoquinone, the synthetic vitamin K, is also scarcely soluble in water. Its effects upon the metabolism of algae are quite similar to those observed with phthiocol. Phthiocol, 2-oxy-3-methyl-1,4-naphthoquinone, occurs naturally in *Bacillus tuberculosis*. Its vitamin K activity is low, but it is soluble in slightly alkaline media. In the following experiments it was added to the cell suspensions in form of a M/250 solution in M/250 disodium phosphate, which has a red color.<sup>1</sup> Its effect on photosynthesis is comparable to that of hydroxylamine. At 25°, pH 6, and 4,000 lux concentrations of about  $10^{-5}$  M phthiocol decrease the rate of oxygen evolution in *Scenedesmus* about 10 per cent.  $10^{-4}$  M phthiocol causes a 90 per cent inhibition. With higher concentrations the inhibition is complete. In alkaline solutions phthiocol forms ionized salts which apparently do not penetrate into the cell. Turning to anaerobic conditions, we find that phthiocol is slowly reduced to the hydroquinone (*cf.* 3). If hydrogen is present, more hydrogen is taken up than by the cells alone; simultaneously the red color disappears. The latter is seen to return in presence of low oxygen partial pressures which do not yet enforce the reversion of the algae to aerobic conditions. The effect of phthiocol upon photoreduction is identical with that of hydroxylamine or phenanthroline, except that much smaller concentrations suffice to obtain it. Fig. 3 shows that a concentration of  $10^{-5}$  M produces a measurable inhibition. With increasing concentrations of phthiocol, the rate of photoreduction decreases to 50 per cent of the normal rate (under conditions of illumination, of course, where the normal rate can still be measured). From then on it remains constant, indicating that now the reaction is not influenced by phthiocol.

<sup>1</sup> With the concentrations used the light absorbed by the colored phthiocol solutions is negligible as compared with that absorbed by the algae. This unimportant complication could be avoided by using red light.

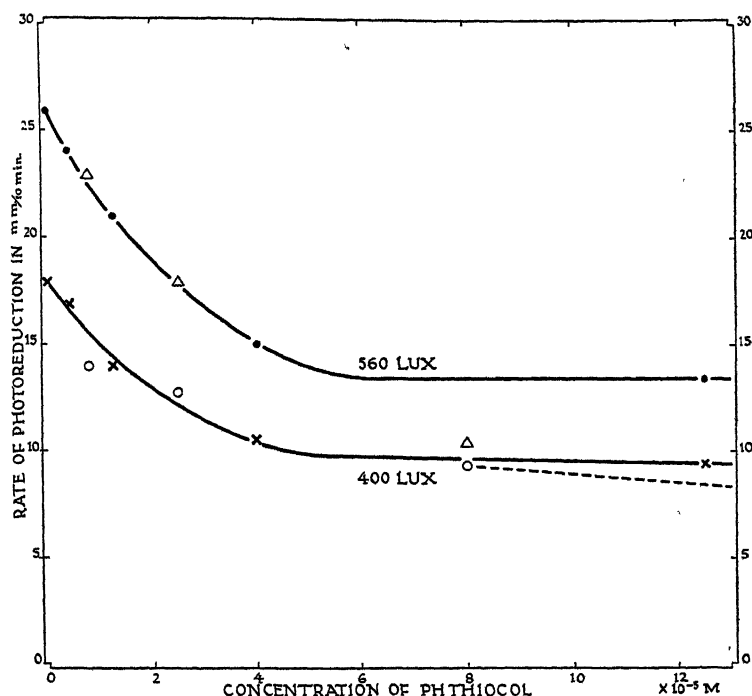


FIG. 3. Inhibition and stabilization of photoreduction in *Scenedesmus* by increasing concentrations of phthiocol. ● — ● rates at 560 lux; △ — △ same a day later. × — × rates at 400 lux; ○ --- ○ same a day later.

TABLE III

*Stabilization of Photoreduction by 2-Methyl-1,4-Naphthoquinone (Vitamin K; Menadiione) and by 2-Methyl-3-Hydroxy-1,4-Naphthoquinone (Phthiocol)*

Time elapsed in the dark	Light intensity	Buffer with 1 per cent alcohol		Normal Buffer	
		Control	Saturated with vitamin K	Control	$1.1 \times 10^{-4}$ M phthiocol
		Rates of photoreduction (—), and of photosynthesis (+), mm./10 min.			
hrs.	lux				
1	560	—9.0	—4.7	—8.0	—5.3
12	560	—11	—3.5	—11	—5.5
12.5	1,015	Reverts +2.0	—7.0	Reverts +1.0	—10
13	4,300	+7.5	—23	+10	—29
27	4,300	+9.0	±0	+10	—3

Those concentrations of phthiocol or of vitamin K which cut the rate of photoreduction down to this lower limit stabilize the reaction against reversion by excess light (Table III). With  $1.25 \times 10^{-4}$  M phthiocol it is possible to

produce true light saturation curves of photoreduction. In the experiment of which the data of Fig. 4 form a part the maximum rate at 30,000 lux (2700 foot-candles) was 113 mm./10 min., that is about three times the rate at which reversion would occur in unpoisoned cells. The first part of the light saturation curve (Fig. 4) found with "stabilized" algae is linear. The linearity is observed

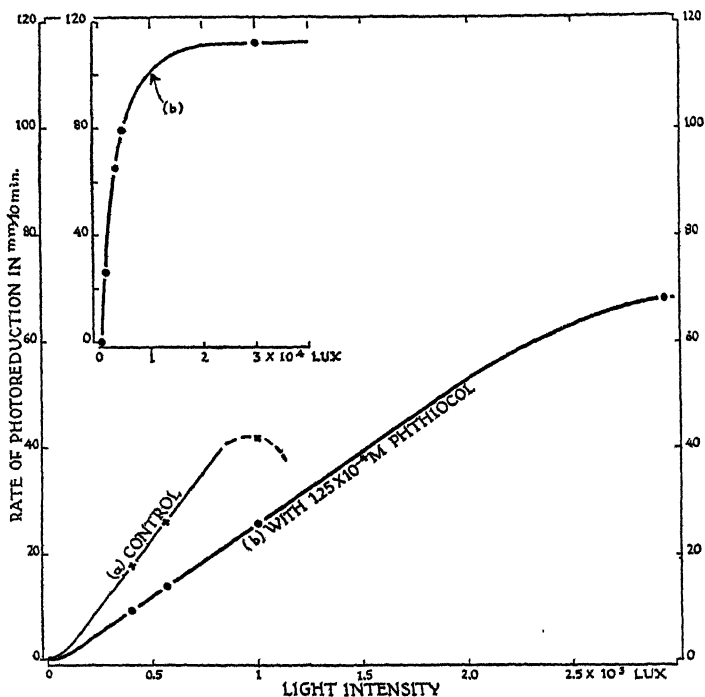


FIG. 4. Light saturation curve for photoreduction stabilized with phthiocol. 0.024 cc. of cells of *Scenedesmus* in 3 cc. of  $M/30$  phosphate buffer pH 6.2. Temperature: 25°. Gas phase:  $H_2$  with 4 per cent  $CO_2$ . Adaptation time: 14 hours.  $\times - \times$  without phthiocol, reverts to photosynthesis at about 800 lux.  $\bullet - \bullet$  with  $1.25 \times 10^{-4} M$  phthiocol. Insert shows the same curve complete in smaller scale.

in the unpoisoned algae until reversion starts, except that the absolute rates here are twice as large. The same concentrations of phthiocol or vitamin K which aerobically stop photosynthesis and anaerobically cut the rate of photoreduction in half while stabilizing the reaction against reversion also inhibit the oxyhydrogen reaction (see the preceding paper). The bleaching of the cells often observed with larger concentrations of vitamin K derivatives (above  $10^{-3} M$ ) in protracted experiments (12 to 24 hours) appears to have nothing to do with the specific anticatalytic effects just described. Once the chlorophyll

has changed color the cells are dead. The first indication of this sort of interference may be the slow increase of the inhibition with time (Tables III and IV).

### 3. Combined Influence of Phthiocol and *o*-Phenanthroline

It should be pointed out that the 1,4-naphthoquinones appear to act upon the metabolism of plants in two ways, either as oxido-reduction catalysts or as substances combining specifically with heavy metal enzymes. Instances of the first type of activity would be the acceleration of respiration and the transfer of hydrogen to oxygen (see preceding paper); of the second, the inhibiting effects paralleling those produced by hydroxylamine or *o*-phenanthroline. The experiments shown in Tables V and VI demonstrate that a combination of *o*-phenanthroline and phthiocol produces no greater effect upon photoreduction

TABLE IV  
*Time Effect on the Inhibition of Photoreduction by Phthiocol*

Suspension of *Scenedesmus* D<sub>3</sub> in (a) M/100 NaHCO<sub>3</sub>, (b) M/25 NaHCO<sub>3</sub>. Gas phase: H<sub>2</sub> with 4 per cent carbon dioxide. Adaptation period: 210 minutes. Temperature: 25°. Time allowed for the reduction of phthiocol: 90 minutes.

Concentration of phthiocol.....	(a)		(b)	
	M/10,000	M/3,000	M/10,000	M/3,000
Time elapsed after adding the poison	Inhibition of photoreduction			
<i>min.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
90	47	50	47	60
720	62	71	55	82

than that obtainable by one of the poisons alone. This speaks in favor of the assumption that both substances stop the activity of the same enzyme system and probably in the same manner.

### 4. The Assimilatory Quotient in the Adapted and "Stabilized" Algae

Rieke has found that in algae like *Scenedesmus* the lowest quantum number for the reduction of one molecule of carbon dioxide with hydrogen is ten (7); that is exactly the same number as obtained in recent years for normal photosynthesis in different laboratories (4-7).<sup>2</sup>

<sup>2</sup> Dorrestein, Wassink, and Katz (8) report an average yield of 12 for photoreduction in *Chromatium*, yet are wondering why students in this country have had the courage once and for all to dismiss as invalid the earlier measurements of Warburg and Negelein. Emerson and Lewis analyzing the procedure of Warburg and Negelein showed clearly that the older measurements are meaningless if one is not sure about the value of the assimilatory quotient, which varies greatly with the experimental conditions in question.



TABLE V

*Combined Influence of Phthiocol and o-Phenanthroline upon Photoreduction*

0.02 cc. of cells of *Scenedesmus* in 4 cc. of M/25 bicarbonate. Gas phase: H<sub>2</sub> with 4 per cent CO<sub>2</sub>. Temperature 25°. Adaptation period: 20 hours. Vessels flushed with fresh gas mixture 5 hours before first measurements. Light intensities: I = 560 lux, II = 4,300 lux;

Concentration of poisons.....	a		b		c		d	
	0.0		1 × 10 <sup>-4</sup> M phthiocol		2.5 × 10 <sup>-4</sup> M o-phenanthroline		= b + c Phthiocol plus o-phenanthroline	
Intensities.....	I	II	I	II	I	II	I	II
Time after poisoning	Rates of photoreduction, mm./min.							
20 min.	-3.9	(+4)	-2.7	(+2.2)	-3.0	-6.4	-2.7	-6.6
18 hrs.	(+0.4)	(+3.0)	-2.0	-6.9	-1.8	-3.0	-2.0	-5.9
22 hrs.	(+0.4)	—	-2.0	—	-1.5	—	-2.0	—
(Concentrations of poisons doubled)								
23 hrs.	(+0.4)	—	-2.0	-6.8	-1.5	-4.6	-2.0	-6.6
32 hrs.	—	—	-1.6	-6.1	-1.4	-4.6	-1.6	-3.6

Test for reversibility. Algae in (a) and (d) washed several times in bicarbonate and acid phosphate, resuspended in bicarbonate as above.

	Rates of photosynthesis at 2,000 lux, mm./min.	
	33 hrs.	+1.43   +1.06
	Rates of photoreduction at 560 lux, mm./min.	
	48 hrs.	-2.30   -0.55

TABLE VI

*Combined Influence of Phthiocol and o-Phenanthroline upon Photoreduction*

0.024 cc. of cells of *Scenedesmus* D<sub>2</sub> in 3 cc. of M/50 sodium bicarbonate. Temperature: 25°. Gas phase: H<sub>2</sub> with 4 per cent CO<sub>2</sub>. Adaptation period: 14 hours. After each change of light intensity the poisoned algae showed a long induction period until the constant rate was attained.

Concentration of poisons.....	0.0	1 × 10 <sup>-4</sup> M o-phenanthroline	4 × 10 <sup>-4</sup> M phthiocol	1 × 10 <sup>-4</sup> M o-phenanthroline and 4 × 10 <sup>-4</sup> M phthiocol
Intensity of illumination	Rates of photoreduction, mm./min.			
lux				
560	-2.3	-1.5	-0.6	-0.6
1,015	-4.6	—	-1.1	-1.4
4,300	Reverts	—	-4.5	-3.0 reverts

In Rieke's measurements of the quantum yield during photoreduction (unpublished), the computations were based on the previously determined assimilatory quotient of *two*, as against *one* under aerobic conditions. The fact that the photochemical evolution of oxygen could be changed to an absorption of hydrogen and back without a shift in the computed yield proves again that the now generally established value of 0.1 for the quantum yield is real and not a result of variations in the quotient.

As long as the light intensity is the only rate-determining factor the rate of the photoreduction is a measure of the quantum yield. We have seen above that a number of poisons halve this rate even at low light intensities. This may happen in two ways: either by a change in the assimilatory quotient, or by an actual drop in the quantum yield. A change of the quotient was not

TABLE VII

*The Assimilatory Quotient in Normal and Stabilized Photoreduction*

*Scenedesmus obliquus* in  $M/20$   $KH_2PO_4$ , half-diluted nutrient medium. Temperature  $25^\circ$ . 14 hours adaptation in pure hydrogen. Flushed with fresh hydrogen 15 minutes before start of experiment. 40 minutes of preliminary illumination to remove internal carbon dioxide. Poisons added 1 hour before introduction of carbon dioxide. 60 c.mm. of carbon dioxide added in form of a boiled solution of sodium carbonate. Duration of experiment 180 minutes.

H <sub>2</sub> absorbed after addition of 60 c.mm. of CO <sub>2</sub> , c.mm.			
Control without CO <sub>2</sub>	Control with CO <sub>2</sub>	$1 \times 10^{-4} M$ o-phenanthroline	$2 \times 10^{-4} M$ phthiocol
-11	-135	-138	-140

The time course of the reaction is shown in Fig. 5.

entirely improbable because in the oxyhydrogen reaction the ratio hydrogen to oxygen falls often from two to one after poisoning with various substances (9). Half the hydrogen is now supplied by intracellular donors.

To know what happens in the poisoned algae, the assimilatory quotient was determined in the manner described in an earlier paper (10). Table VII shows that the total amount of hydrogen absorbed for a given quantity of carbon dioxide does not change in the presence of the stabilizing poisons. The value of the quotient remains two. Fig. 5 presents the time course of the complete reduction of 60 c.mm. of carbon dioxide by hydrogen without and with poison added. What has changed is the rate only, that is, under the prevailing circumstances, the quantum yield.

### 5. Iodoacetamide

The inhibition of photosynthesis by iodoacetamide has been described by Kohn (11). Our experiments on photoreduction with this type of poison,

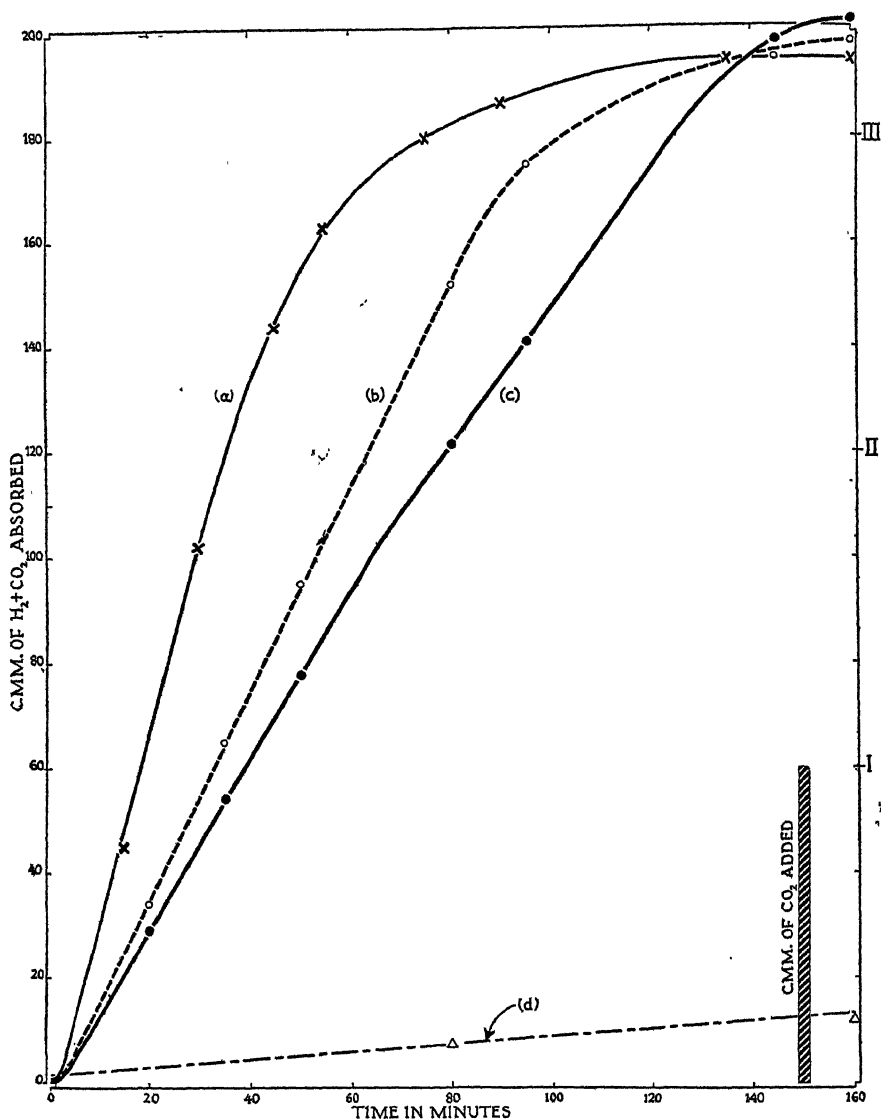


FIG. 5. Assimilatory quotient during photoreduction in presence of *o*-phenanthroline or of phthiocol. Reduction of 60 c.mm. of carbon dioxide with 120 c.mm. of hydrogen. *Scenedesmus obliquus*, (a) no poisons added, (b) with  $10^{-3}$  M *o*-phenanthroline, (c) with  $2 \times 10^{-4}$  M phthiocol, (d) blank without carbon dioxide.

supposed to combine with sulfhydryl groups in the enzyme proteins, were not very satisfactory because, as Tables VIII and IX show, the effect of a certain concentration continues to increase with time. This is understandable, since

the reactions of the cell material with iodoacetamide are irreversible. The inhibition of photoreduction sets in, and proceeds, faster than that of photo-

. TABLE VIII

*Effects of Iodoacetamide upon Photosynthesis, Adaptation, and Photoreduction*

0.033 cc. of cells of *Scenedesmus* in M/30 phosphate buffer pH 6. Temperature 25°. Gas phase: H<sub>2</sub> with 4 per cent CO<sub>2</sub>. Poison added 10 minutes before start of measurements.

	1	2	3	4
	Control	10 <sup>-3</sup> M iodoacetamide	10 <sup>-4</sup> M iodoacetamide	Control
I	(a) Rate of photosynthesis at 2,000 lux, mm./min.			
	+1.1	+0.16	+1.1	+1.1
	(b) Adaptation. Total pressure changes, mm. in 15 hrs.			
	-48	+1.0	-13	-59
	(c) Rate of photoreduction at 900 lux, mm./min.			
First 5 min.....	-2	+2	±0	±0
After 15 min.....	-26	±0	+1	-24
II. Iodoacetamide added in vessel 4 after adaptation. 10 <sup>-4</sup> M iodoacetamide				
Rate of photoreduction, mm./min.				
900 lux.....	-34	±0	±0	-7
3,000 lux.....	—	±0	-4	-34
16 hrs. later				
3,000 lux.....	-32	+1	+1	±0

TABLE IX

*Effect of Iodoacetamide on Photoreduction*

0.038 cc. of cells in 4 cc. of phosphate buffer pH 6.5. Temperature 25°. Gas phase: H<sub>2</sub> with 4 per cent CO<sub>2</sub>. Adaptation time: 16 hours. Poison added 30 minutes before first illumination.

Light intensity	Rate of photoreduction, mm./min.		
	Control	0.5 × 10 <sup>-4</sup> M iodoacetamide	1 × 10 <sup>-4</sup> M iodoacetamide
560 lux	-2.7	-2.0	-1.2
2 hrs. dark			
560 lux	-3.1	-0.8	-0.4
1,000 lux	-6.0	-1.8	-0.8
3,000 lux	(-0.1 Reversion)	-4.0	-2.0
40,000 lux	—	(-0.2 Reversion)	(-0.8 Reversion)

synthesis, but this might be due not to differences in the enzyme system attacked, but to the general differences between aerobic and anaerobic conditions. As long as the inhibition has not proceeded too far, the rate of photoreduction in the poisoned algae responds linearly to an increase of the light

intensity. Because the rates are diminished the intensity threshold for reversion under the influence of light is higher than normal. There is, however, no true stabilization against reversion. In this respect the results obtained with iodoacetamide are comparable to the effect due to poisoning with dinitrophenol or to lack of carbon dioxide.

TABLE X  
*Inhibition of Adaptation by Chloretone*

0.03 cc. of cells of *Scenedesmus* D<sub>3</sub> in 5 cc. of M/10 carbonate buffer. Temperature: 25°. Chloretone added as saturated aqueous solution. Gas phase: air, later H<sub>2</sub>.

Dilution of chloretone solution.....	Control	1/250 saturated	1/50 saturated	1/10 saturated
(a) Metabolic rates, mm./min.				
Respiration.....	-0.53	-0.49	-0.43	-0.27
Photosynthesis				
4,300 lux.....	+7.0	+7.0	+7.0	+5.8
560 lux.....	+1.3	+1.2	+1.3	+1.2
(b) H <sub>2</sub> absorbed during 12 hrs. in hydrogen, mm.				
	-129	-117	-101	(+21)
(c) Rate of photoreduction in successive periods of 10 min., mm./min.				
Photoreduction				
1,000 lux				
First 10 min.....	-0.3	-0.8	-0.2	+0.5
Second 10 min.....	-0.4	-1.2	+0.4	+0.9
Sixth 10 min.....	-2.4	+1.1	+1.1	+1.1
(d) Photoreduction after 12 hrs. incubation with 2 mg. glucose. Illumination in successive periods of 5 min. light alternating with 5 min. dark. Rates, not counting the dark intervals, mm./min.				
Photoreduction				
560 lux				
First period.....	-0.0	-1.6	-1.4	-0.0
Second period.....	-0.2	-2.6	-1.4	-0.2
Sixth period.....	-0.2	-3.0	-0.2	-1.0
Sum total in 30 min. light, mm.....	-6.0	-71.0	-34.0	+5.0

## 6. Chloretone

Concentrations of chloretone which diminish the rate of respiration in *Scenedesmus* D<sub>3</sub> to about 50 per cent have little influence upon photosynthesis, particularly at low light intensities, but inhibit strongly the adaptation to photoreduction. These observations parallel those concerning the cyanide inhibition. This is interesting because chloretone is a typical "narcotic" drug, and yet it acts here rather specifically. With slightly smaller concentrations, a slow but normal adaptation cannot be prevented. Upon illumination, however, the reversion to photosynthesis occurs in the slightly narcotized algae much earlier and at lower intensities than in the controls (Table X). This

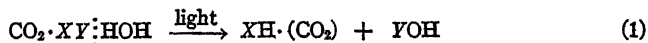
latter effect would suffice to explain why an adaptation was not observed with the highest concentrations of chloretone. Among the enzymatic systems the hydrogenase (or one link of the hydrogen transfer system) appears to be the most sensitive one to the narcotic action of chloretone. The early reversion notwithstanding, small concentrations of this poison were found to produce often an acceleration of the hydrogen consumption, particularly at the beginning of an illumination period, as shown in Table X. This latter effect of chloretone becomes clearly demonstrable when the algae have been incubated with glucose, a treatment which is known to diminish or sometimes even to abolish the reaction with free hydrogen. We may assume that chloretone inhibits the utilization of internal hydrogen donors more strongly than that of molecular hydrogen.

#### DISCUSSION

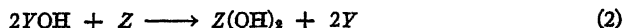
The earlier experiments with hydroxylamine proved that in presence of a "stabilizing" dose of poison the rate of photoreduction can attain a light saturation value considerably above the normal reversion threshold, but that at lower light intensities the rate is smaller than normal. The maximum inhibition averages 50 per cent and increases slowly with time. Merely the fact of an incomplete inhibition was considered theoretically important, the limit of 50 per cent attributed to chance. The data of the present paper leave little doubt, however, that the ratio one-half is not accidental, but that it has a meaning; that it is not due to a variable difference in reaction velocities between the poison-sensitive and poison-fast photoreduction, but to a stoichiometric change concerning the reactions of the photochemical products. Hence that part of the equations and diagrams (2) describing the qualitative difference between the normal and the "stabilized" photoreduction must be modified so as to account for the quantitative aspect as well.

The complete discussion given in a recent review (2) cannot be repeated here. We shall make use of only some of the equations presented there. They are given the same numbers as in the said article. Under the assumption that the poison inhibits the production of "peroxide," the difference between the labile and the stabilized photoreduction was described by the following equations:

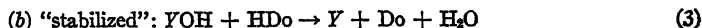
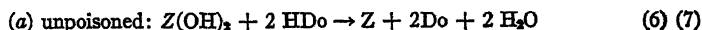
Photochemical reaction:



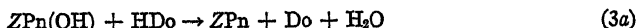
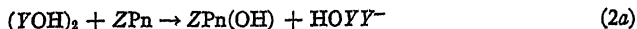
Formation of a "peroxide:"



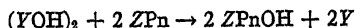
Reduction by hydrogenase



To account for the loss of exactly one-half of the photochemical products equation (3) has to be replaced by (2a) and (3a). The poison Pn is assumed to combine with catalyst Z in such a manner that the "peroxide" cannot be formed but that one-half of the oxidized photoproducts is transferred to the hydrogenase, while the remaining half disappears in reaction (8).



An interesting condition is that a reaction of the type



must be excluded, and that instead of symbolizing the first oxidized product by YOH we would have to write—as shown in reaction (2a)—HOYYOH, -YYOH, and YY. This would imply a two step oxidation reduction process in the mechanism of the light reaction.

Though formally our problem can be solved in this manner, another solution is also possible, based not on the loss of one-half but of all the original photochemical products. In photosynthesis one molecule of oxygen is evolved per molecule of carbon dioxide reduced to carbohydrate. After adaptation of the algae to hydrogen one molecule of oxygen taken up in the dark causes the reduction of only one-half molecule of carbon dioxide (9). Accordingly, the second way to account for half the quantum yield in the stabilized photoreduction is to assume that the normal photochemical products, reduced substances XH and oxidized substances YOH, react back completely in an oxidation reaction which is coupled with the reduction of carbon dioxide in the same manner as the oxyhydrogen reaction. Since not less than one hydrogen atom (or electron) can be transferred in an elementary step, or per quantum, the photochemical process must have proceeded at least two steps forward (if not four) before a back reaction can be expected to cause the transfer of a hydrogen atom with the yield one-half. The way "down" must be different from the way "up." Otherwise each molecule of water decomposed in the light should cause the transfer of one hydrogen when reformed in the course of the back reaction. The analogy with the oxyhydrogen reaction requires that the reappearance of every second molecule of water only is coupled with the transfer of one hydrogen atom from the hydrogenase to the carbon dioxide complex.

#### SUMMARY

It is known that with increasing concentrations of hydroxylamine the rate of photoreduction in the alga *Scenedesmus* drops to about one-half of the normal rate. From then on photoreduction remains insensitive to hydroxylamine. The present experiments prove that this strange effect is not specific for hydroxylamine. It can be produced with substances having quite different

chemical properties, such as *o*-phenanthroline, 2-methyl-1,4-naphthoquinone (vitamin K), or 2-oxy-3-methyl-naphthoquinone (phthiocol). Once the rate of photoreduction has been brought down to the limit of exactly one-half by a sufficient dose of any one of these substances, the reaction is also stabilized against reversion under the influence of strong light. At saturation intensities the rate of the stabilized photoreduction may be several times that at which the unpoisoned cells revert to photosynthesis. The ratio of one-half between the rates of the stabilized and the normal photoreduction is found at very low light intensities. This indicates a change in the photochemical process. Since the assimilatory quotient remains unaltered, it is the quantum yield which is cut in half under the influence of the poisons. To explain these observations it is assumed that either just one-half of the primary photo-products are lost, or that they react back entirely while causing a reduction of carbon dioxide in a way similar to that brought about by the oxyhydrogen reaction in the dark.

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# NATURE OF THE GROWTH FACTOR FOR THE COLORLESS ALGA PROTOTHÉCA ZOPFII

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The detailed studies of Barker (1) on the metabolism of *Prototheca zopfii* demonstrated that development of this organism did not take place in media containing a suitable carbon source and inorganic or amino nitrogen only. Yeast autolysate or some other source of complex organic material was found requisite for development. The quantity of yeast extract required for growth, however, was very small. In media containing low concentrations of this material development of the organism could be greatly increased by the addition of an ammonium salt, such as ammonium chloride. Although this substance could not serve as a substitute for yeast autolysate, quantitative studies showed that a large portion of the nitrogenous cell material could be synthesized from the ammonia nitrogen. The maximum cell yield of *Prototheca* was shown to be a complicated function of the amounts of yeast autolysate and ammonia nitrogen available. From these experiments Barker concluded: "Yeast autolysate or some other complex material is indispensable for the development of this alga."

It seemed logical to investigate the possibility, indicated in the findings of Barker, that yeast autolysate contributed one or more essential growth factors. A detailed study of this phase was attractive because the identification of such factors would make it possible to investigate their function in the metabolism of this organism.

## Material and Methods

*Organism.*—The strain of *Prototheca zopfii* used was No. 7322, one of the several maintained in the pure culture collection at the Hopkins Marine Station. It was selected from the group on the basis of its rapid development in a simple liquid medium.

*Medium.*—Cultures of the organism were maintained on yeast agar containing 2 per cent glucose.

For studies on the growth factor requirements of the organism, a basal liquid medium of the following composition proved satisfactory:

	per cent
Glass-distilled H <sub>2</sub> O	
NH <sub>4</sub> CL.....	0.10
MgSO <sub>4</sub> .....	0.02
KH <sub>2</sub> -Na <sub>2</sub> HPO <sub>4</sub> (pH 7.0).....	0.20
Glycerol.....	0.50

For growth factor studies it is imperative that all tests for substances, functioning as nutrilites for a particular organism, be carried out in a basal medium which is as simple as possible and yet contains *all* other elements necessary for growth of the organism. The composition of the glycerol mineral medium fulfills these requirements closely enough, since in the absence of yeast extract or of the active components of this material practically no growth occurs, whereas the addition of these materials gives rise to a profuse development.

Glycerol was chosen as a carbon source in preference to glucose because glycerol is not acted upon by *Prototheca* under anaerobic conditions. While glucose is aerobically converted into cell material and carbon dioxide, anaerobically it is quantitatively fermented into lactic acid (1). Therefore, the use of glycerol has the great advantage over glucose in that the acidity of the medium resulting from the growth of the organism on glycerol will be solely that arising from fermentation of stored carbohydrate products. This obviates the necessity of the addition of large quantities of calcium carbonate to the culture medium as a buffer. Glycerol has the added advantage as a substrate for growth factor tests in that it does not undergo decomposition or polymerization when sterilized in the presence of phosphates as does glucose. The products resulting from heat sterilization of sugar have been found to influence the growth of microorganisms, exerting a toxic effect on some and acting as growth-promoting substances for others (Stanier (2), and Fulmer, Williams, and Werkman (3)). Finally, glycerol can much more easily be obtained free from minute amounts of organic impurities, which might serve as growth factors, than can carbohydrates.

To determine if autoclaving resulted in a serious destruction of growth factors, duplicate series of varying concentrations of yeast autolysate were prepared using sterile autolysate and heat-sterilized medium base. One series was autoclaved at 15 pounds pressure for 20 minutes and both series were inoculated with equal amounts of a dilute *Prototheca* suspension. Cell yield determinations for each series indicated conclusively that under the conditions employed in the tests the growth promoting substances are quite heat-stable and that sterilization may be safely accomplished by autoclaving.

Glassware used for all growth factor experiments was scrupulously cleaned with cleaning solution and at least four final rinsings with distilled water.

*Culture Methods.*—Although *Prototheca* is capable of carrying on an anaerobic metabolism it is totally unable to develop under strictly anaerobic conditions (1). In tests for the activity of growth factors it is essential that the conditions of aeration approach the optimum in order that the growth of the organism will be a function of the amounts of the growth substances present rather than being restricted by a limited oxygen supply. Quantitative experiments were therefore carried out with cultures in shallow layers in rotating bottles. Unless otherwise stated these cultures were incubated at 30° C. for a period of 7 days.

Although satisfactory growth curves were obtained with cultures growing in liquid media dispensed in 50 ml. volumes in Florence flasks of 125 ml. capacity, such curves

indicated reduced oxygen tension to be a limiting factor in the growth of *Prototheca* in flasks. This was especially true at the higher concentrations of growth-promoting substances. To obtain more nearly optimum aerobic conditions under which to grow *Prototheca*, a method of culture was devised which would allow the surface of the liquid medium to be very large as compared with its volume. This was accomplished by using 25 ml. volumes of medium in 150 ml. narrow mouth glass bottles having an inside diameter of approximately 5 cm. When the bottles were placed on their sides the greatest depth of the liquid was about 1 cm. To further insure uniform aerobic conditions the bottles were placed in a rolling machine and continuously rolled at the rate of about 18 R.P.M.

In order to compare the growth of *Prototheca* in 25 ml. amounts of medium in bottles with that in 50 ml. amounts in flasks, graduated series of concentrations of yeast autolysates were set up in each type of culture vessel and incubated for a period of 10 days. Cell yield measurements for each series are recorded in Fig. 1. These

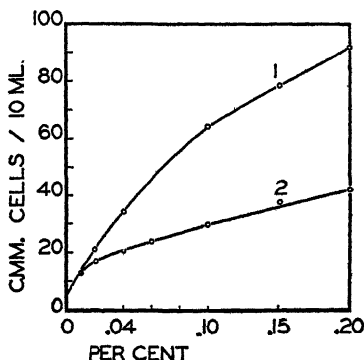


FIG. 1. Comparison of the growth of *Prototheca* in bottles (1), and in flasks (2), in media containing varying concentrations of yeast autolysate.

clearly demonstrate that growth of *Prototheca* in flasks under semi-aerobic conditions is definitely inferior to that produced under aeration such as is obtained in shallow layers continuously rolled. Further experiments showed that with the higher yeast extract concentrations double the amount of growth could be obtained in bottles in 1 week as that obtainable in flasks during a 2 week period of incubation.

*Quantitative Determination of Cell Yield.*—Determination of cell yield was accomplished by centrifuging aliquot portions of the cultures. Ordinarily, 10 ml. were centrifuged in Hopkins vaccine tubes for 10 minutes at 2700 R.P.M. The results so obtained are quite reproducible and of an accuracy sufficient to show a linear relationship between cell volume and the amount of growth factor in the lower concentrations of this material.

#### *Fractionation of Yeast Autolysate and Activity of the Fractions*

Since Barker had shown yeast autolysate to contain substances essential for the growth of *Prototheca* this material was selected as a source from which to

attempt the isolation of the active constituents. Preliminary extraction tests using ether, chloroform, and 95 per cent ethanol as solvents were carried out on aliquot samples of yeast autolysate adjusted to pH 2.5, 7.0, and 9.0. The autolysate used in these tests was prepared by letting pressed yeast autolyze in the presence of chloroform according to the method of Willstätter (4). Additional extractions using ether and 95 per cent ethanol as solvents were carried out in a Soxhlet extraction apparatus on a dehydrated yeast extract powder (Difco standardized). Appropriate amounts of the soluble and insoluble fractions obtained for each pH value for each solvent were mixed with the basal medium to provide a wide series of media for each fraction. These were inoculated with equal amounts of a *Prototheca* suspension. Cell volume determinations made on aliquot samples from each culture showed that the growth-promoting substances for *Prototheca* are not extracted by ether or chloroform from acid, neutral, or alkaline solutions, as in each case the total activity remained in the insoluble fraction. Cell yield values obtained in the series of media containing the various ethanol fractions showed that the nutritives for the alga are alcohol-soluble. In addition, growth of the organism in the fractions obtained in the alcoholic extraction of yeast autolysate which had been adjusted to pH 9.0 indicated that the growth-promoting substance had undergone some destruction.

*Comparison of Active Material with Known Growth Factors.*—The solubility characteristics of the active substance in the various solvents tested, strongly suggested the possibility of its association with the group of B vitamins known at the time these investigations were undertaken. More specifically, its sensitivity to alkali indicated a striking similarity to the properties possessed by vitamin B<sub>1</sub> (thiamin). Consequently, a number of experiments were set up with the mineral glycerol medium, to which a graded series of concentrations of three members of the B group (thiamin, riboflavin, and nicotinic acid) had been added. Growth determinations showed that only those media which contained vitamin B<sub>1</sub> permitted development of *Prototheca* and that the further addition of riboflavin and nicotinic acid did not result in any greater cell yield than was obtained with thiamin alone. The maximum growth in the presence of thiamin alone approximated closely that previously observed in the presence of yeast extract. Comparative experiments using the basal medium enriched with yeast autolysate and with thiamin in different concentrations corroborated this. Additional experiments, in which combinations of low concentrations of yeast autolysate and thiamin were used, showed the activity of the two materials to be additive. Serial subcultures in the glycerol medium with added vitamin B<sub>1</sub> have shown *Prototheca* capable of the same level of growth for many transfers.

These tests consequently established the chemical nature of the factor which is responsible for the activity of yeast autolysate in the growth of *Prototheca*. It can be asserted that the only nutritive required by this alga is thiamin. Its

activity is very high; concentrations as low as  $3 \times 10^{-11}$  M permit a detectable growth of the alga. The response to increasing concentrations of thiamin is shown in Table I and part of the data has been plotted in Fig. 2. This figure shows that the relationship between thiamin concentration and cell yield is virtually linear to  $1 \times 10^{-7}$  M thiamin. At this concentration growth is practically as heavy as the maximum obtainable with larger amounts of the vitamin. Considerably higher concentrations of vitamin B<sub>1</sub> seem to show a tendency to

TABLE I  
*Growth of Prototheca in Various Concentrations of Thiamin*

Molar concentration of thiamin	Cell yield	Molar concentration of thiamin	Cell yield
	<i>mm.<sup>3</sup> per 10 ml.</i>		<i>mm.<sup>3</sup> per 10 ml.</i>
$1 \times 10^{-10}$	7	$5 \times 10^{-8}$	56
$3 \times 10^{-10}$	9	$8 \times 10^{-8}$	80
$1 \times 10^{-9}$	12	$1 \times 10^{-7}$	96
$3 \times 10^{-9}$	15	$3 \times 10^{-7}$	110
$1 \times 10^{-8}$	20	$1 \times 10^{-6}$	105
$2 \times 10^{-8}$	32	$1 \times 10^{-5}$	100
$3 \times 10^{-8}$	40	$3 \times 10^{-5}$	97

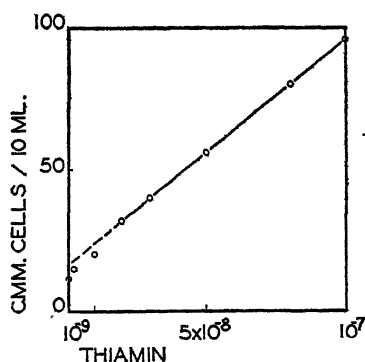


FIG. 2. Relationship between molar concentration of thiamin and cell yield.

exert a slight inhibition. Although the accuracy of determination of cell yield at this cell concentration is insufficient to stress this depressant effect, it may be stated that such results were consistently obtained and that Schopfer (5) has made similar observations.

#### *Thiamin and Its Components As Growth Factors*

*Thiamin Requirements of Plants and Animals.*—The general need for thiamin—as a vitamin for animals, as a necessary growth factor for many microorganisms, and as a hormone (6) for plant cells—shows that it plays an important rôle in the growth of the most diverse types of cells.

The elucidation of the chemical configuration of thiamin and its simple quantitative cleavage into the thiazole and the pyrimidine portions has made possible detailed studies concerning the replaceability of thiamin by its major constituents and by laboratory-synthesized analogues of these constituents. This approach has revealed that both the higher and lower animals require the complete thiamin molecule. Even the protozoa not obviously derivable from algae (ciliates, trypanosomes) behave in this manner. On the other hand many plant tissues and plant-like microorganisms are less exacting and are capable of fulfilling their thiamin requirements from a mixture of the two components of the thiamin molecule as effectively as from the whole molecule. Some of the microorganisms are even able to satisfy their growth requirements from the thiazole or the pyrimidine component supplied singly. In this connection it is interesting to note that certain of the fungi considered as typical plant parasites, resemble the animals in that they require the entire thiamin molecule for growth.

The microorganisms can thus be divided into five groups according to their relationship to thiamin and to its components:

(a) Organisms requiring the whole thiamin molecule: The Lwoffs (7 to 10) have shown the ciliate *Glaucoma piriformis* and three species of the flagellate *Strigomonas* to resemble the animals in their vitamin B<sub>1</sub> requirements; Robbins (11) reports ten fungi of the genus *Phytophthora* to likewise require the complete thiamin molecule.

(b) Organisms needing thiamin or both of its components in equimolar concentrations: Knight (12) showed *Staphylococcus aureus* to need thiamin or its components for growth, neither part alone functioning in this capacity. Similar findings were made for the molds *Phycomyces blakesleanus* and *Phycomyces nitens* by Schopfer and Jung (13) and by Robbins and Kavanagh (14) and by Sinclair (15). Schopfer (5) found the fungus *Pilaira moreaui* to require both components also. Lwoff and Dusi (16 to 18) broadened the known list of organisms capable of utilizing mixtures of both components with their findings for the flagellates *Polytoma caeca* and *Chilomonas paramecium*. Robbins and Kavanagh (19) included the yeast *Torula laurentii*, and the basidiomycetes *Ustilago violacea* and *U. scabiosae* were added by Schopfer and Blumer (20).

(c) Types of organisms requiring only the pyrimidine component: In the long list of fungi whose growth factor requirements were investigated by Schopfer (21) we find five species of *Rhodotorula* and one of *Dematium* representing the yeasts, and the zygomycetes *Absidia ramosa*, *Parasitella simplex*, and *Pilaira anomala* which are capable of satisfying their growth factor requirements from the pyrimidine component alone. Robbins and Kavanagh (22) found two representatives of *Pythium* and one of *Phytophthora* to include in this type.

(d) The fourth group of organisms, those capable of utilizing the thiazole portion alone, includes *Mucor ramanianus* (Müller and Schopfer (23)), and the protozoa *Polytoma caudatum* (Lwoff and Dusi (17)), and *Polytoma ocellatum* (Lwoff and Dusi (24)). It may be pointed out that these two species of protozoa are related to the Chlamydomonas group of green algae and thus can be expected to possess thiamin requirements characteristic of the plant-like organisms.

(e) The fifth type comprises a large group of bacteria, yeasts, molds, and algae which are capable of normal development in the absence of any external source of thiamin or its components.

*Identification of the Growth Factors for Prototheca zopfii*

Although thiamin was found to satisfy the growth factor requirements of *Prototheca*, further tests were made to determine whether this organism needs the whole vitamin molecule, a mixture of the thiazole and pyrimidine components, or one of the components alone as the active principle.

TABLE II

*Cell Yield in Mm.<sup>3</sup> per 10 Ml. of Prototheca Cultures Grown in the Presence of Different Concentrations of Thiamin and of Various Pyrimidine Preparations*

Molar concentration	Thiamin	Pyrimidine						
		3	3 B	4	5	5 W	6	7 M
$5 \times 10^{-6}$	104	8	9	8	7	7	4	10
$1 \times 10^{-6}$	94	8	9	8	7	8	4	7
$5 \times 10^{-7}$	96	8	9	8	7	8	4	8
$1 \times 10^{-7}$	94	8	7	8	7	7	4	8
$5 \times 10^{-8}$	86	8	7	8	7	9	4	7
$1 \times 10^{-8}$	32	8		8	7	9	4	7
$5 \times 10^{-9}$	17	8	7	9	7	8	4	7
$1 \times 10^{-9}$	6	6	7	8	7	8	4	5
Control	2							

3 = 2-methyl-4-amino-5-aminomethyl pyrimidine

3 B = 2-methyl-4-amino-5-aminomethyl pyrimidine

4 = 2-methyl-4-amino-5-hydroxymethyl pyrimidine

5 = 2-methyl-4-amino-5-chloromethyl pyrimidine

5 W = 2-methyl-4-amino-5-chloromethyl pyrimidine

6 = 2-methyl-4-amino-5-aminomethyl pyrimidine

7 M = 2-methyl-4-amino-5-ethoxy pyrimidine

I. G. Farbenindustrie

Buchman (supplied by Dr. James Bonner)

I. G. Farbenindustrie

I. G. Farbenindustrie

Winthrop

I. G. Farbenindustrie

Merck

*Growth of Prototheca on the Thiazole and on the Pyrimidine Component.*—In experiments conducted to determine the ability of the alga to grow on thiazole or pyrimidine alone, media containing various concentrations of five thiazole preparations<sup>1</sup> and seven pyrimidine analogues<sup>1</sup> were made up in glycerol mineral medium and sterilization was accomplished by autoclaving.

Cell yield measurements in media containing pyrimidine alone showed about equal but very slight development for all samples, with one exception, and in no case did an increase in concentration over a 5,000-fold range cause an appreciable increase in cell yield. Data for these growth tests are presented in Table II.

<sup>1</sup> These preparations were kindly supplied by the I. G. Farbenindustrie, Winthrop Chemical Company, Merck and Company, and Dr. James Bonner and Dr. E. R. Buchman of the California Institute of Technology.



The results obtained with four different samples of 4-methyl-5-hydroxyethyl thiazole and one sample of the benzoic acid ester of the same compound are recorded in Table III. They show that, while the benzoic acid ester was without any activity whatever, the effects of the four "natural" thiazole compounds on the growth of *Prototheca* were far from comparable. Cell yields ranged from an amount equal to that obtained in the controls to very nearly the maximum volume obtainable from growth on the whole vitamin. Since the thiazoles used in these tests were different preparations of the same compound, the purity of those permitting heavy growth was not above suspicion. Additional growth

TABLE III

*Cell Yield in Mm.<sup>3</sup> per 10 ML. of Prototheca Cultures Grown in the Presence of Different Concentrations of Thiamin and of Various Thiazole Preparations*

Molar concentration	Thiamin	Thiazole				
		1	1 W	1 M	1 B	2
$5 \times 10^{-6}$	104	70	86	84	21	2
$1 \times 10^{-6}$	94	24	42	90	10	2
$5 \times 10^{-7}$	96	18	28	84	6	2
$1 \times 10^{-7}$	94	9	12	51	2	2
$5 \times 10^{-8}$	86	7	8	33	3	2
$1 \times 10^{-8}$	32	4	3	12	3	2
$5 \times 10^{-9}$	17	4	5	2	3	2
$1 \times 10^{-9}$	6	3	2	2	2	2
Control	2					

1 = 4-methyl-5-hydroxyethyl thiazole

I. G. Farbenindustrie

1 W = 4-methyl-5-hydroxyethyl thiazole

Winthrop

1 M = 4-methyl-5-hydroxyethyl thiazole

Merck

1 B = 4-methyl-5-hydroxyethyl thiazole

Buchman (supplied by Dr. James Bonner)

2 = 4-methyl-5-hydroxyethyl thiazole benzoic acid ester

I. G. Farbenindustrie

tests showed three of the thiazoles to be seriously contaminated to different extents with pyrimidines or substances capable of acting in that capacity. Contaminating substances in the fourth thiazole were of sufficiently low concentration to be detectable only when used in excessively high concentrations; concentrations up to  $10^{-5}$  M failed to provide a satisfactory source of growth factors for *Prototheca*.

*Growth of Prototheca on Mixtures of the Natural Thiazole and Pyrimidine Components.*—Experiments were carried out to determine the ability of *Prototheca* to grow in a series of media containing graded concentrations of mixtures of the purest thiazole and one of the natural pyrimidine preparations. The results of one experiment are summarized in Table IV. They show unequivocally that *Prototheca zopfii* is able to utilize a combination of the two com-

ponents in place of the whole thiamin molecule and that the components are required in equimolar proportions. The cell yield is determined by the component of the thiamin molecule that is present in the smaller amount.

The data obtained in these experiments give rise to some further comments. Both in the absence and in the presence of very low concentrations of thiazole the growth of *Prototheca* appears to be due to some extent to the amount of pyrimidine present. This suggests the presence of a limited supply of thiazole in either the medium, the inoculum, or as a contaminant of the pyrimidine. The presence of thiazole as an impurity of the pyrimidine should have resulted in increased growth in the excessively high concentrations used to test this component alone and, therefore, can be ruled out.

TABLE IV

*Cell Yield in Mm.<sup>3</sup> per 10 Ml. of Prototheca Cultures Grown in the Presence of Different Concentrations of Mixtures of the Purest Thiazole and Pyrimidine*

Molar concentration		Pyrimidine 3B							
		0	$1 \times 10^{-10}$	$3 \times 10^{-10}$	$1 \times 10^{-9}$	$3 \times 10^{-9}$	$1 \times 10^{-8}$	$3 \times 10^{-8}$	$1 \times 10^{-7}$
Thiazole 1 B	$3 \times 10^{-7}$	2	3	5	12	22	42	92	94
	$1 \times 10^{-7}$	2	3	5	12	22	42	100	96
	$3 \times 10^{-8}$	2	3	5	12	21	44	92	94
	$1 \times 10^{-8}$	2	3	5	12	21	41	54	48
	$3 \times 10^{-9}$	2	3	5	10	17	22	22	22
	$1 \times 10^{-9}$	2	3	4	10	14	15	14	15
	$3 \times 10^{-10}$	2	3	4	10	12	11	11	12
	$1 \times 10^{-10}$	2	3	4	8	11	12	11	11
	0	2	3	4	8	9	10	9	10

The occurrence in nature of thiazole unaccompanied by an equivalent quantity of pyrimidine is apparently so rare that the chemicals used in the basal medium would seem most unlikely as a source of thiazole contamination. If thiazole were introduced with the inoculum it can be inferred that the cells used for this purpose must have contained an excess of thiazole. Special experiments, however, to test the storage of thiazole by *Prototheca* gave negative results.

The higher concentrations of the mixtures allow a total cell yield closely approximating the maximum growth obtainable in cultures growing in media containing the complete vitamin or yeast autolysate. A number of experiments have shown that maximum cell yield is obtained at a concentration approximately  $3 \times 10^{-8}$  M for each component of the thiamin molecule. It is interesting to note that this is about one-tenth of the concentration necessary when the whole molecule is used. This observation is in line with that of Schopfer who found *Phycomyces blakesleeana* to require approximately twice as much thia-

min as a mixture of the two components in order to give comparable maximum yields.

Since the alga cannot grow unless supplied with both pyrimidine and thiazole, *Prototheca zopfii* represents another member of the previously mentioned group (b).

#### SUMMARY

Barker's study on the nutritive requirements of *Prototheca zopfii* indicated that this colorless alga fails to grow in the absence of small amounts of yeast extract. A study of the growth factor requirements of *Prototheca* has shown that the active constituent of yeast extract necessary for the growth of this organism is thiamin (vitamin B<sub>1</sub>). Thiamin can fully replace the complex yeast material and allows, in the basal medium used, a maximum cell yield in concentrations of  $1-3 \times 10^{-7}$  M.

Thiamin as such, however, is not essential for the growth of *Prototheca zopfii*. The alga can develop equally well if supplied with both the thiazole and pyrimidine constituents of this vitamin. These appear to be needed in equimolar proportions. Maximum cell yield is obtained with  $3 \times 10^{-8}$  M concentrations of the two components.

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# STUDIES ON THE METABOLISM OF THE COLORLESS ALGA *PROTOTHECA ZOPFII*

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## INTRODUCTION

The first studies dealing with the physiology of the colorless alga *Prototheca zopfii* were those of Barker (1, 2) who demonstrated that the metabolism of this organism was essentially of an oxidative nature. He found that while it was capable of carrying on an anaerobic metabolism in that it could ferment glucose quantitatively to lactic acid, it was totally unable to develop under strictly anaerobic conditions. In this respect its metabolism may be compared with that of mammalian muscle tissue.

Barker made a detailed study of the metabolism of this alga in regard to its nutritional requirements for growth and to its ability to utilize a great variety of simple carbon compounds as the sole substrate.

Of the carbohydrates tested, only the monosaccharides were utilized by *Prototheca*. All of the fatty acids, with the exception of formic and isovaleric acid, appeared adequate as carbon sources, as did many of the alcohols and ketones. None of the nitrogen-containing compounds (glycine, asparagine, glucosamine, ethylamine, or yeast autolysate) was found to serve as a carbon source. The most surprising result was the fact that not a single substituted or dicarboxylic acid tested by Barker would serve as a utilizable substrate.

The study of the carbon nutrition was approached experimentally in two ways. Culture experiments in which the substance under investigation constituted the main carbon source of the medium showed what compounds could serve for growth. The effect of the addition of various organic substrates upon the oxygen consumption by suspensions of non-growing cells was studied with the manometric technique of Warburg-Barcroft.

In all cases of the oxidation of utilizable compounds, Barker was able to express the relationship between the quantity of substrate, oxygen, and carbon dioxide participating in the reaction in terms of a balanced chemical equation having simple stoichiometric relations. This relationship showed conclusively that those compounds which are attacked by *Prototheca* are not oxidized completely to carbon dioxide and water, but that a considerable fraction, from 50 to 80 per cent, is converted into a primary assimilation product having an over-all composition corresponding to that of a carbohydrate which is stored in the cells, probably as glycogen. Barker's experiments showed that the process of assimilation of simple organic substrates by this alga proceeds

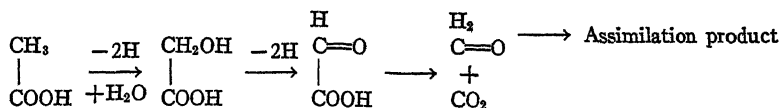
in two distinct stages. The first he found to consist of a very rapid oxidative conversion of the substrate into carbon dioxide and the stored material. The second stage comprises a slow decomposition of this primary assimilation product and its subsequent transformation into many different organic substances which make up the cell material.

This was the first convincing demonstration that, during the respiration of simple organic compounds by non-proliferating organisms, assimilation processes occur to an unexpectedly large extent. That a large oxidative assimilation is not restricted to the metabolism of a colorless alga was soon evidenced by the studies of Giesberger (3) on various *Spirillum* species and by Clifton (4, 5) on *Pseudomonas calcoacetica* and *Escherichia coli*.

Since the oxidation of such simple compounds as acetic acid by *Prototheca* resulted in so extensive a synthesis of carbohydrate-like materials, it suggested the possibility of an experimental approach to the problem of the mechanism of synthetic processes in general. The stoichiometric relationship seemed to indicate that the substrate would be partially oxidized, giving rise to intermediate products from which the synthesis to carbohydrate could proceed directly. In that case, a study of the behavior of the various organic compounds that could be postulated as being intermediate products in the synthesis of carbohydrate from acetic acid should reveal the general pathway of the metabolic reactions involved in the biochemical synthesis. This would appear to be a most fruitful approach, particularly in view of the fact that a vast number of studies have clearly demonstrated that the production of carbohydrate is one of the most important aspects of photosynthesis.

*Prototheca* is a member of the family Oocystaceae, order Chlorococcales, class Chlorophyceae (Chlorophyta), and represents an alga devoid of chlorophyll, and hence unable to produce organic cell materials from carbon dioxide as the sole carbon source. Nevertheless, in a primary assimilatory process such as is characteristic of the metabolism of *Prototheca*, the synthesis of carbohydrate from a simple organic compound shows a certain similarity to the photosynthetic reaction. Moreover, the great economy of carbon assimilation is not entirely restricted to photosynthesis, as is evidenced by the fact that Barker found *Prototheca* capable of assimilating such a large percentage of the carbon of a single substrate. One might expect the further conversions of the primary assimilatory product into numerous cell materials to proceed by much the same types of mechanism in both *Prototheca* and in the green plants, so that a detailed study of the metabolism of the former would ultimately aid in understanding that of the latter. Finally, heterotrophic organisms in general carry out synthetic reactions of various sorts. In view of the well established similarity of biochemical mechanisms in the most divergent types of organisms, a study of carbohydrate synthesis from acetate by *Prototheca* should be of decided value in contributing to a general clarification of such syntheses.

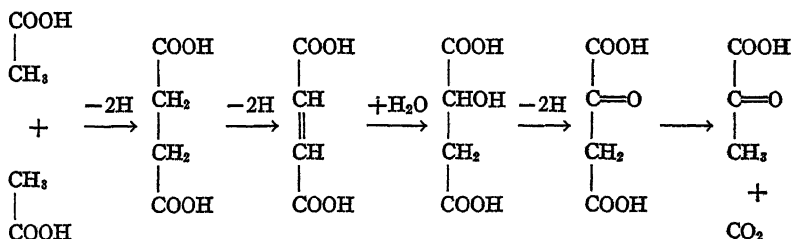
However promising the outlook, Barker's experiments appeared, at that time, to lead up a blind alley. This can best be appreciated by considering the oxidative metabolism of *Prototheca* in the presence of acetate. Two main pathways for the decomposition of this metabolite can be postulated. One would be through successive oxidations to glycolic and glyoxylic acids and its subsequent decarboxylation (Bernhauer (6)).



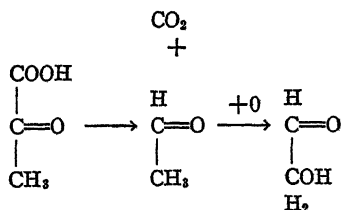
Synthesis of carbohydrate could then take place from the "formaldehyde," more or less in accordance with von Bayer's concept of carbohydrate formation in photosynthesis.

If *Prototheca* were to oxidize acetate in this manner it should follow that both glycolic acid and glyoxylic acid could be metabolized. Barker was forced to rule out this series of reactions, however, since *Prototheca* was incapable of oxidizing either intermediate.

The second manner in which acetate could be oxidized proceeds by way of succinic, fumaric, and oxalacetic acids, followed by decarboxylation to pyruvic acid (Thunberg (7)).



The further fate of pyruvic acid could be postulated to lead, through a second decarboxylation, to acetaldehyde from which, by oxidation to glycolaldehyde, carbohydrate might be formed by condensation:



This scheme too, would satisfy the experimental values for the relationship between acetate used, oxygen consumed, and carbon dioxide produced. But

this series proved no more tenable than did the first, for the intermediates here involved were likewise not metabolized when tested by Barker.

The identification of thiamin as the growth factor for *Prototheca* (8) made it possible to investigate its specific function in the oxidative metabolism of this organism. In the course of this work a number of new facts were discovered which gradually led to a skeptical attitude with respect to the general validity of some of Barker's findings. A reinvestigation of certain phases of his work was then undertaken which resulted in a much more satisfactory picture of the metabolism of the experimental organism. This, in turn, made it possible to carry out some preliminary experiments in connection with the assimilation problem proper.

### *Material and Methods*

*Organism.*—The strain of *Prototheca zopfii* used in these investigations was No. 7322, one of several maintained in the pure culture collection of the Hopkins Marine Station, and is the same strain as that used in the studies on the growth factor requirements of this organism (8).

*Medium and Methods of Culture.*—Cultures of the organism were maintained on yeast agar containing 2 per cent dextrose.

"Normal" or vitamin-sufficient cells for use in making manometric measurements were grown on plates of the yeast dextrose agar medium incubated at 30°C. for 48 hours. The cells were washed once or twice by centrifuging and resuspended in sterile tap water at pH 7.0, or in M/15 phosphate buffers of the desired pH.

To obtain thiamin-deficient cells it is necessary to grow the organism in a medium in which thiamin is the limiting factor. Such cells were obtained by using a glycerol mineral medium (8) to which thiamin was added in sufficient amounts to allow good growth with limiting concentrations of the vitamin. The cells were grown in several rotating bottles, each containing 25 ml. volumes of glycerol mineral medium to which thiamin had been added. At the end of a 6 day period of incubation at 30°C. the cultures were pooled, centrifuged, washed once or twice, and resuspended in neutral sterile tap water, or in M/15 phosphate buffers, in a concentration suitable for use in making manometric measurements.

### FUNCTION OF THIAMIN IN THE METABOLISM OF *PROTOTHECA ZOPFII*

Thiamin has been found to be essential for the normal development of *Prototheca* (8). Therefore the hypothesis that a growth factor or a vitamin represents the functional (active or prosthetic) group of an enzyme without which normal metabolism cannot proceed, leads to the assumption that the alga needs carboxylase and uses this enzyme in its metabolism but is unable to synthesize carboxylase unless supplied with thiamin or its component parts.

It was generally held, at the time the present work was undertaken, that the sole function of carboxylase was to catalyze the decarboxylation of  $\alpha$ -keto acids. Furthermore, since the only known connection of thiamin with enzymes was its occurrence in carboxylase, it seemed logical to conclude that the de-

carboxylation of  $\alpha$ -keto acids formed an integrant part of the metabolism of *Prototheca zopfii*.

*Prototheca* is capable of using sugars as a substrate. From the accumulated data on the sugar metabolism of various organisms it was logical to assume pyruvic acid to be an intermediate substance in the decomposition of sugar. Therefore, carboxylase, and hence thiamin, could be expected to be needed in the metabolism of this alga. A serious difficulty was presented, however, by the fact that Barker had claimed *Prototheca* to be incapable of decomposing pyruvic acid. Consequently, Barker's experiments on the utilization of pyruvic acid by *Prototheca* were repeated.

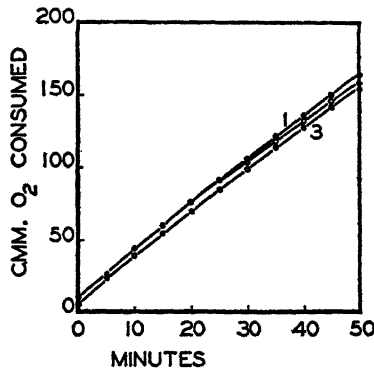


FIG. 1. Oxidation of pyruvate at pH 7.0 by non-deficient cells of *Prototheca*: (1) autorespiration; (2) 0.01 mM Na pyruvate; (3) 0.01 mM Na pyruvate plus 10  $\gamma$  thiamin.

The oxygen consumption of 2.0 ml. volumes of normal cells in neutral tap water was measured by the manometric technique of Warburg-Barcroft, in vessels containing KOH in the center well. In the absence of an added substrate which the cells are capable of utilizing, the rate of oxygen consumption by such suspensions of non-proliferating cells is relatively low, and represents the rate of oxidation of cellular materials, such as reserve carbohydrates. The addition of a utilizable substrate greatly increases the rate of oxygen consumption (see also Barker (2)). Numerous experiments demonstrated that the addition of sodium pyruvate to such suspensions did not result in an increase in the rate of oxygen utilization. Even the addition of thiamin did not cause an acceleration of oxygen uptake. Fig. 1. represents the results obtained in one typical case.

The results were in complete agreement with the experiments reported by Barker. As a consequence of these unequivocal findings, Barker's conclusion that *Prototheca* was unable to decompose pyruvic acid was believed to be correct.

In view of the recognized function of carboxylase, it was difficult to understand why *Prototheca* needed thiamin as a growth factor and yet was unable



to decompose pyruvic acid. This strongly indicated that in the case of *Prototheca* thiamin might be used not for the synthesis of carboxylase, but for the synthesis of an enzyme concerned in the decomposition of organic compounds other than  $\alpha$ -keto acids. The most characteristic metabolites for *Prototheca* are the unsubstituted fatty acids, especially acetic, as is attested by the abundant development of this organism in vinegar casks (Janke (9)). This natural occurrence of the alga indicates that acetic acid is a suitable enrichment substrate for *Prototheca*. Furthermore, the careful and extensive studies of Barker had shown that *Prototheca* was unable to utilize any but the fatty acids. Therefore, it seemed possible that thiamin might function in the decomposition of the fatty acids, and in particular in the oxidation of acetic acid. Consequently experiments were designed to determine whether thiamin functioned in the oxidation of fatty acids by *Prototheca*.

The method of approach was based on the technique developed by Lwoff (10) in his investigations on the rôle of blood in the nutrition of trypanosomes and later utilized by Hills (11) in his studies on the part played by thiamin, a growth factor for *Staphylococcus aureus*, in the metabolism of this organism.

Hills showed that staphylococci, grown with minimal amounts of thiamin, consumed oxygen in the presence of pyruvate as a substrate at a very low rate. The rate of oxygen consumption was immediately increased by the addition of minute amounts of thiamin. Cells grown with optimum amounts of thiamin were capable of a rapid decomposition of pyruvate and their oxygen consumption was not affected by the addition of the vitamin. These experiments made it clear that the oxidation of pyruvate by *Staphylococcus aureus* requires an enzyme which the organism can rapidly synthesize from thiamin. The fact that the anaerobic decomposition (decarboxylation) of pyruvic acid by "thiamin-deficient" cells was also greatly and immediately increased by the addition of thiamin to the cell suspension, showed that the main, if not only, function of the vitamin, in the metabolism of *Staphylococcus aureus*, is its conversion into carboxylase.

The above examples illustrate what could be expected from an application of Lwoff's "starvation" methodology to a study of the metabolism of *Prototheca*. Those substrates, for which a special thiamin-containing enzyme is required, would be decomposed slowly by cells that had been grown in media deficient in this vitamin. The addition of small amounts of thiamin to suspensions of such deficient cells should result in an immediate and rapid synthesis of the limiting enzyme system with a subsequent increase in the rate of utilization of the substrate.

#### *Experiments with Thiamin-Deficient Cells*

To determine if thiamin functioned in the oxidation of fatty acids by *Prototheca* the following experiment was carried out.

Thiamin-deficient cells were obtained by growing the organism in glycerol mineral medium to which  $1 \times 10^{-7}$  M thiamin had been added. Two ml. samples of the non-proliferating cells resuspended in neutral tap water were placed in the vessels of Warburg-Barcroft manometers. Each vessel contained KOH in the center well and a gas phase of air. Ten  $\mu$ g. of thiamin were added to the suspension in each of two vessels. Measurement of oxygen consumption in the absence of added substrate showed the cells without added thiamin to have a low rate of oxygen uptake. On the other hand, the cells supplied with thiamin showed a rate of oxygen utilization approximately twice that of the starved cells. This difference in autorespiration indicates that the addition of thiamin allows the cells to utilize some of their stored materials more rapidly, suggesting that the lack of the vitamin restricted the metabolism of these substances.

The addition of 0.01 mM of sodium acetate caused a rapid and nearly identical increase in oxygen consumption by the cells deficient in thiamin and by those to which thiamin had been added as well. In this case, therefore, thiamin had no effect whatever on the oxidation of acetate.

The addition of 0.01 mM of dextrose presented an entirely different picture. The thiamin-deficient cells consumed oxygen at a very low rate; the quantity utilized during the first hour was exactly the same as in the control. The extra oxygen consumption in the suspension containing sugar, which began at that time, increased slowly thereafter. The behavior of deficient cells was plainly different from that of normal cells in the presence of dextrose. Cells having access to thiamin showed an immediate increase in oxygen consumption in response to the addition of dextrose. The marked difference in rate of decomposition of dextrose by the deficient cells with and without added thiamin clearly indicated that thiamin is essential to *Prototheca* in its metabolism of dextrose. The results of a representative experiment showing the effect of thiamin on the oxidation of acetate and of dextrose are presented in Fig. 2.

Apparently *Prototheca* could oxidize a representative of the fatty acids without benefit of thiamin, but it did need this substance, or its components, for growth and for the utilization of a sugar. The failure of thiamin to affect the oxidation of acetic acid indicated that there was no reason to ascribe to it a function in the metabolism of fatty acids. Its pronounced influence on sugar metabolism supported the possible relation of thiamin to the decomposition of pyruvic acid. It was almost certain that this  $\alpha$ -keto acid would occur as an intermediate substance in the degradation of sugar by *Prototheca zopfii*. The strongest support for this contention was the extensive production of lactic acid from glucose under anaerobic conditions (Barker (1)). The entire body of evidence, amassed by Emden, Meyerhof, and others (12), made it reckless to suggest that the formation of this hydroxy acid would not proceed, in the case of *Prototheca*, by the well established mechanism of the reduction of pyruvic acid. In sharp contrast with these theoretical deductions was the experimental evidence, showing that pyruvate was not metabolized by *Prototheca*.

*Experiments on the Effect of pH on the Decomposition of Pyruvic Acid*

In all cases, determinations of the ability of *Prototheca zopfii* to utilize a given substrate had been carried out in neutral solutions. There is much evidence to show that cells are more freely permeable to undissociated molecules than to ions.

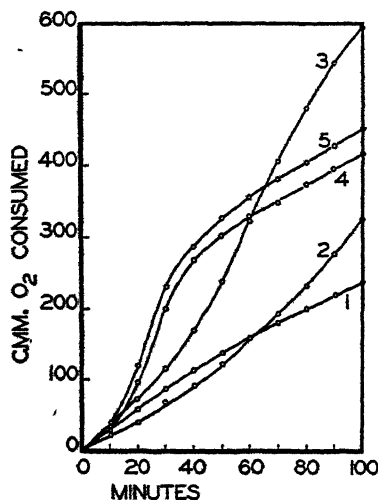


FIG. 2

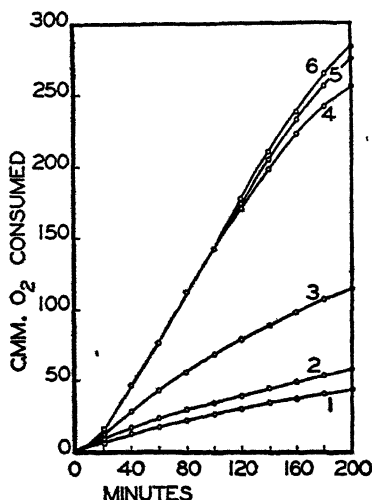


FIG. 3

FIG. 2. Effect of thiamin on oxidation of dextrose and acetate by vitamin-deficient cells of *Prototheca* at pH 7.0: (1) autorepiration; (2) 0.01 mM dextrose; (3) 0.01 mM dextrose plus 10  $\gamma$  thiamin; (4) 0.01 mM Na acetate; (5) 0.01 mM Na acetate plus 10  $\gamma$  thiamin.

FIG. 3. Effect of pH on pyruvic acid oxidation: (1) autorepiration at pH 4.0; (2) 0.01 mM pyruvic acid at pH 6.0 to 5.5; (3) at pH 5.5 to 4.5; (4) at pH 4.5 to 4.0; (5) at pH 4.0 to 3.5; (6) at pH 3.5 to 3.0.

The work of Osterhout, Collander, and others has shown that in a neutral environment weakly dissociating acids and bases penetrate cells more rapidly than do strongly dissociating ones. Conditions that suppress the dissociation of a substance have a tendency to increase its penetration. There are indications in the literature that many organisms are unable to metabolize strong acids except in an acid environment. A striking example of this phenomenon is furnished by the photochemical nitrate reduction carried out by the green alga, *Chlorella*. Warburg and Negelein (13) found this reaction to take place only in solutions containing undissociated nitric acid. The anaerobic decomposition of pyruvic acid by yeast has also been shown to proceed most rapidly in an acid medium (14). Pyruvate is decomposed by cell-free yeast juice at a pH of about 6.0. Living yeast cells, however, fail to cause any appreciable decomposition of the keto acid until the acidity of the suspending medium is increased

to a value below pH 4.0. Such behavior may be explained as a result of the nature of the cellular membrane of certain cells which allows acids to penetrate only in the form of undissociated molecules.

Therefore experiments were conducted to test the possibility that pyruvic acid might be attacked when the suspension liquid was maintained at an acid reaction.

Vitamin non-deficient cells were obtained in the usual manner and resuspended in sterile tap water at a pH of 7.0. One-half ml. of the suspension, containing 12 mm.<sup>3</sup> of cells, was measured into each of six Warburg vessels containing 1.5 ml. of a series of phosphate solutions at pH values ranging from pH 6.0 to 3.5. Each vessel contained NaOH in the center well and air as the gas phase. Autorepiration, determined over a period of 90 minutes, was quite similar throughout the range of hydrogen ion concentrations used. The lower pH values, however, did have a slight stimulatory effect on the rate of oxygen consumption, the cells at pH 3.5 using 43 mm.<sup>3</sup> of oxygen during the initial 90 minute period, as against 35 mm.<sup>3</sup> at pH 6.0.

The addition of 0.01 mm. of pyruvic acid to the suspension of cells at pH 6.0 lowered the pH to 5.5 and caused a very slight initial increase of oxygen uptake which soon returned to a rate identical with that for the autorepiration (13 mm.<sup>3</sup> per 50 minutes). At this stage the pH had risen to 5.7 as a result of the decomposition of a small amount of added pyruvic acid. At pH 5.5 the addition of the same amount of acid caused an increase in acidity to pH 4.5 with a subsequent initial rate of oxygen consumption of 38 mm.<sup>3</sup> per 50 minutes which gradually fell off to a rate of 24 mm.<sup>3</sup>. Again at this point, the decomposition of a portion of the pyruvic acid had resulted in a decrease in acidity of the suspension; pH determinations showed it to be 5.2. The addition of the keto acid to the suspensions of organisms at pH 4.5, 4.0, and 3.5, now lowered to pH 4.0, 3.5, and 3.0 respectively, presented a distinctly different picture. In these three cases the rate of oxygen utilization immediately rose to 80 mm.<sup>3</sup> per 50 minutes. This high rate was maintained until the pyruvic acid had been decomposed. Final pH determinations of these three suspensions showed that their acidity had returned to the initial values. The oxygen consumption for each suspension, from the time the pyruvic acid was added, is shown in Fig. 3.

Since the metabolism of pyruvic acid proceeded in nearly an identical manner at pH 4.0, 3.5, and 3.0, the highest value was chosen for use in all subsequent experiments in order to avoid any possible injury to the cells by an environment too strongly acid. Although Barker (1) reported an increase in hydrogen ion concentration to have an adverse effect on the rate of dextrose decomposition by *growing* cultures of *Prototheca*, further tests have shown that at pH 5.0, at least, the decomposition of dextrose by non-proliferating cells is certainly not inhibited but rather that the acid environment exerts a stimulatory effect.

This experiment was the first to demonstrate that *Prototheca* does possess enzyme systems capable of decomposing pyruvic acid. From the data recorded in Fig. 3, it is seen that a hydrogen ion concentration greater than that corresponding to pH 4.5 is necessary in order that pyruvic acid may be made available to the intracellular enzyme systems.

*Effect of Thiamin on Pyruvate Decomposition*

To determine whether thiamin is needed in the metabolism of *Prototheca* in the rôle ascribed to carboxylase, vitamin-deficient cells, with and without added thiamin, were tested for their ability to decompose pyruvic acid in an acid environment.

Deficient cells grown in glycerol mineral medium containing  $1 \times 10^{-8}$  M thiamin were centrifuged, washed twice in M/15 primary phosphate solution adjusted to pH 4.0 by the addition of  $\text{H}_2\text{SO}_4$ , and resuspended in the "buffer" in a concentration suitable for use in Warburg measurements. Two ml. of the suspension, containing 32 mm.<sup>3</sup> of cells, were introduced in each of the vessels and the rate of oxygen consumption measured. The first vessel received no added substrate and, therefore, its rate of oxygen uptake is a measurement of the autorepiration of stored cellular materials. The cells in the second vessel received 0.01 mm of pyruvic acid. With the addition of the acid the rate of oxygen consumption was increased, indicating that the vitamin-deficient cells were able to decompose pyruvic acid. To the suspension of cells in the third vessel were added 10  $\mu\text{g}$ . of thiamin. The addition of the vitamin to the deficient cells caused an immediate but slight increase in the rate of autorepiration. Addition of 0.01 mm of pyruvic acid to the cells now supplied with thiamin, caused a rate of oxygen uptake 2.5 times the maximum obtainable with the vitamin-deficient cells to which no thiamin was added. Suspensions of cells treated in a manner identical with that of deficient cells, except that they had been grown in a medium containing an optimum amount ( $1 \times 10^{-6}$  M) of thiamin, were able to decompose pyruvic acid at a high rate without added vitamin. The vitamin-sufficient cells showed no increase in rate of oxygen consumption on the addition of the same amount of thiamin which caused a 2.5-fold increase in the rate with the deficient cells. The data obtained in one representative experiment are presented in Fig. 4.

Extensive investigations of the effect of thiamin on decomposition of pyruvic acid by suspensions of non-proliferating, vitamin-deficient cells have shown that the addition of the vitamin in general causes a 2.0 to 3.0-fold increase in the rate of oxygen consumption. The percentage of increase is dependent on the degree of vitamin deficiency and on the age of the "insufficient" cells.

Organisms grown in media containing  $1 \times 10^{-8}$  M vitamin B<sub>1</sub> tend to show a greater increase in rate of oxygen consumption on the addition of thiamin, while respiring pyruvic acid, than do cells grown in  $3 \times 10^{-8}$  M vitamin B<sub>1</sub>. Likewise, cells grown for 96 hours in media containing  $1 \times 10^{-8}$  M vitamin B<sub>1</sub> tend to show a greater response than do cells grown in similar media for 72 hours. It is assumed that the older cells are more vitamin "starved" than are the younger cells, since the older cultures contain more cells per unit volume and, therefore, the available vitamin has been distributed to a larger number of organisms. Numerous tests have shown the addition of 10  $\mu\text{g}$ . of thiamin to 2.0 ml. suspensions of vitamin-deficient cells to provide an arbitrary but entirely adequate amount of the vitamin to insure maximum rates of metabolism under all conditions of thiamin deficiency of the cells.

*Evidence for the Occurrence of Pyruvic Acid As an Intermediate Product in the Metabolism of Prototheca*

With the finding that *Prototheca zopfii* can utilize pyruvic acid the apparent discrepancies in the metabolism and growth requirements of the alga appeared to be solved. Thiamin was shown to immediately affect the metabolism of

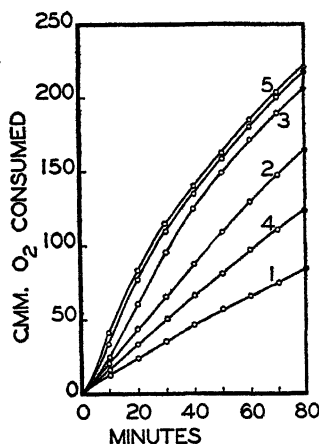


FIG. 4

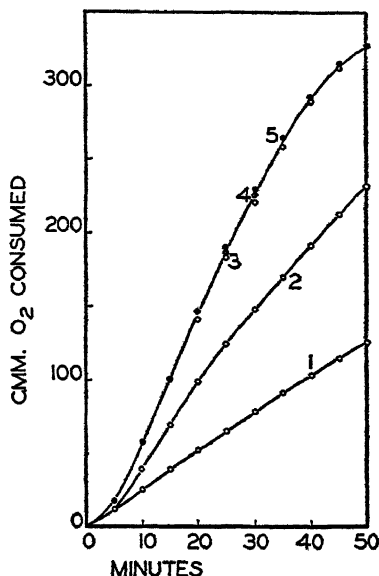


FIG. 5

FIG. 4. Effect of thiamin on pyruvic acid oxidation: (1) autorespiration of deficient cells; (2) deficient cells plus 0.01 mM pyruvic acid; (3) deficient cells plus 0.01 mM pyruvic acid plus 10  $\gamma$  thiamin; (4) autorespiration of non-deficient cells; (5) non-deficient cells plus 0.01 mM pyruvic acid; (6) non-deficient cells plus 0.01 mM pyruvic acid plus 10  $\gamma$  thiamin.

FIG. 5. Effect of pH on lactic acid decomposition: (1) autorespiration; (2) 0.01 mM lactic acid at pH 6.0; (3) at pH 5.0; (4) at pH 4.5; (5) at pH 4.0.

the alga in the presence of sugar and pyruvic acid, but not in the presence of acetate. Therefore, the conservative ideas of the function of the vitamin, or its components, as building blocks for the carboxylase, appear applicable in the case of *Prototheca*.

Lactic acid was tested and found to be attacked by non-proliferating cells of *Prototheca*. This hydroxy acid also was decomposed by the cells only in an acid environment.

Lactic acid ( $K=0.031$ ) is not so strong an acid as is pyruvic ( $K=0.56$ ), and consequently is not so highly dissociated in aqueous solutions. Therefore it would follow

that *Prototheca* suspensions might decompose lactic acid in an environment having a hydrogen ion concentration lower than that required to permit the decomposition of pyruvic acid. The data contained in Fig. 5 indicate that such might be the case. A hydrogen ion concentration corresponding to pH 5.0 will permit a maximum rate of oxygen consumption with lactic acid as a substrate while a pH below 4.5 is necessary for the maximum rate of oxidation of pyruvic acid.

The rate of oxygen utilization of thiamin-deficient cells metabolizing lactic acid could also be increased by the addition of thiamin although the stimulatory effect of the vitamin was not so great as in the case of pyruvic acid. While the addition of thiamin caused a 2.0 to 3.0-fold increase in the rate of pyruvate decomposition, the rate for lactic acid decomposition was increased about 1.4 times. This difference finds a ready explanation in that the decomposition of lactic acid, with pyruvic acid as an intermediate, requires an oxygen uptake for the conversion of the hydroxy to the keto acid. This reaction should be unaffected by vitamin B<sub>1</sub> (carboxylase). The oxygen utilized in this conversion, however, would be included in the rate of oxygen consumption measured. Therefore, the portion of the oxygen consumption which could be expected to be directly influenced by carboxylase would be much less in the decomposition of lactic acid than in the decomposition of pyruvic acid.

The assumption that thiamin functions in the rôle of carboxylase in the normal metabolism of *Prototheca zopfii* is further substantiated by observations on cultures of the alga growing in glycerol media. Pyruvic acid was found to accumulate in the culture medium containing insufficient amounts of the vitamin, whereas not a trace of this substance could be detected in cultures that had grown in the presence of an optimum supply of the growth factor. This is in line with the observations of Platt and Lu (15) and others that pyruvic acid accumulates in tissues and body fluids of animals deprived of vitamin B<sub>1</sub> and also with the finding of Peters (16, 17) that avitaminotic brain tissues of pigeons oxidize pyruvic acid at a subnormal rate.

The experimental evidence that pyruvic acid accumulates in glycerol cultures growing in the presence of suboptimal concentrations of thiamin clearly indicates the formation of the keto acid as an intermediate product in the oxidation of glycerol and demonstrates the difficulty of vitamin B<sub>1</sub>-deficient cultures in disposing of pyruvic acid.

#### *Effect of Thiamin on the Metabolism of Acetate by Prototheca*

Up to this point, studies on the metabolism of vitamin-deficient cells of *Prototheca zopfii* have shown the oxidation of glucose, pyruvic acid, lactic acid, and glycerol to be markedly increased by the addition of small amounts of vitamin B<sub>1</sub>. Since pyruvic acid may be assumed to occur as an intermediate product in the oxidation of all these compounds, and has been demonstrated to accumulate during the "oxidation" of glycerol by thiamin-starved cells,

the results so far presented do not offer any indication that thiamin participates in the decomposition of substances other than  $\alpha$ -keto acids. This is in complete harmony with the hitherto accepted function of carboxylase.

However, later experiments on the oxidation of acetate by non-proliferating, vitamin-deficient cells grown in the presence of  $1 \times 10^{-8}$  M thiamin showed the rate of oxidation of this substrate to be materially accelerated by the addition of thiamin. The results of five experiments, presented in Table I, appear contradictory to previous findings (see Fig. 2). The cells tested in the earlier experiments had been grown in media containing  $1 \times 10^{-7}$  M thiamin and, therefore, cannot be considered to have been so deficient as the cells which were produced in the presence of  $1 \times 10^{-8}$  M thiamin. It would appear, therefore, that a vitamin deficiency is manifest *first* in the metabolism of

TABLE I

*Effect of Thiamin on the Oxidation of Acetate by Suspensions of Thiamin-Deficient, ( $1 \times 10^{-8}$  M), Cells of *Prototheca zopfii**

Age of culture	O <sub>2</sub> utilization		Increase
	With thiamin	Without thiamin	
<i>hrs.</i>	<i>mm.<sup>3</sup> per 50 min.</i>	<i>mm.<sup>3</sup> per 50 min.</i>	<i>per cent</i>
72	117	85	138
72	137	95	144
90	137	87	157
96	100	65	154
96	75	47	160

pyruvic acid, and only later with acetate. Also it should be stated that the deficiency is more pronounced in the metabolism of pyruvic acid; a two- to three-fold stimulation was found with pyruvic acid as a substrate as against an average 1.5-fold increase with acetate.

The somewhat similar stimulatory effect of thiamin on vitamin-deficient cells oxidizing lactic acid was interpreted on the basis that pyruvic acid occurs as an intermediate product in the decomposition of the hydroxy acid and that, therefore, thiamin functions in the oxidation of the keto acid. Is it probable or possible that a similar explanation may be found to interpret the results obtained with acetate?

The mechanism of the oxidation of acetic acid is still unknown. However it is not improbable that some  $\alpha$ -keto acid might be involved as an intermediate product in the decomposition of this simple fatty acid. From a consideration of the schemes for oxidation of acetate proposed in the introduction, either pyruvic acid or glyoxylic acid can be postulated to appear as intermediate products. In that event the effect of thiamin on acetate oxidation would be



similar to its function in the oxidation of lactate, glycerol, or dextrose. That is, thiamin would act in a secondary rôle.

The observation that thiamin can effect the oxidation of acetic acid by *Prototheca* is important in view of the demonstration by Quastel and Webley (18, 19) that vitamin B<sub>1</sub> appears to be essential for the oxidation of acetic acid by an unknown species of bacterium.<sup>1</sup> This organism, when grown on media containing suboptimal amounts of thiamin, responded strongly to additions of vitamin B<sub>1</sub>. This stimulation was found to be particularly pronounced if the vitamin was added in the presence of magnesium and potassium ions (Mg<sup>++</sup> and K<sup>+</sup>).

On the basis of their results, Quastel and Webley have given an involved explanation of this combined vitamin and metal ion effect without, apparently, envisaging the possibility that the response might simply be due to thiamin functioning in the decomposition of intermediate products in the nature of  $\alpha$ -keto acids. At first sight their data might seem to effectively rule out the function of thiamin in relation to  $\alpha$ -keto acids. The stimulation of the rate of oxygen uptake for acetate appears to be much more pronounced than for pyruvate. While the rate of oxygen consumption in acetate oxidation is raised from 20.5 mm.<sup>3</sup> of oxygen per hour per milligram of dried bacteria ( $Q_{O_2}$  20.5), to 63.1 by the addition of K, Mg, and thiamin, the oxygen uptake with pyruvate is increased only from  $Q_{O_2}$  19.9 to 37.1.

This difference in response, of course, is not a very solid argument against the participation of thiamin in the oxidation of pyruvate because the two substrates are *not* in the same "state of oxidation." As an example let us compare the effects obtained in the respiration of lactate and pyruvate. The maximum  $Q_{O_2}$  obtained on the addition of thiamin and metal ions was 37.1 for pyruvate oxidation, while for lactate the  $Q_{O_2}$  was 72. It is most significant that the addition of metal ions alone can increase the lactate  $Q_{O_2}$  from 35 to 53 while similar additions have no effect on the pyruvate  $Q_{O_2}$  which remains at about 20. On the other hand, the addition of thiamin alone raises the lactate  $Q_{O_2}$  from 35 to 52. The maximum increase in  $Q_{O_2}$  due to the addition of vitamin B<sub>1</sub> is therefore 17 for both lactate and pyruvate. Since Quastel and Webley have shown pyruvate to accumulate in suspensions of deficient cells fed lactate in the absence of thiamin, it may be asserted that the identical increase in  $Q_{O_2}$ , due solely to vitamin B<sub>1</sub> results from decomposition of pyruvate in both the lactate and pyruvate oxidation. A similar situation can be shown to exist in the case of succinate and fumarate.

In order to obtain vitamin-deficient cells Quastel and Webley grew their organisms on a deficient medium composed of Difco peptone, agar, and NaCl made up in distilled water and autoclaved for 1 hour at a pH of 9.0 to reduce the vitamin content. The

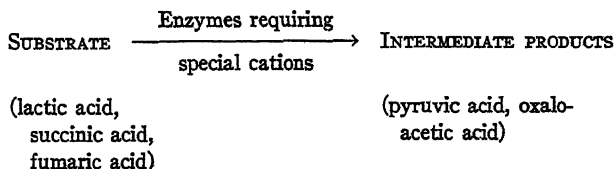
<sup>1</sup> Quastel and Webley claim the organism they used to be a propionic acid bacterium, *Bacterium acidi propionici*. This is most improbable, however, because it grows rapidly, aerobically, and is capable of growth in the absence of sugar or lactate as a substrate. Krebs and Eggleston (20) using the same strain, reported the organism incapable of producing propionic acid.

medium was then filtered through cotton and autoclaved again. As a result of this method of preparation it is obvious that the medium must have been deficient in most metals as well as in vitamin B<sub>1</sub>. Bacterial cells, grown in such an environment, consequently would themselves be deficient in various metals known to play an important rôle in the activity of a number of enzyme systems. That this was so is shown by the fact that if the deficient bacteria were incubated aerobically in the presence of Mg and K for a short time and afterwards thoroughly washed, the oxidative powers of suspensions of such cells were increased by the addition of vitamin B<sub>1</sub>, but not by the further addition of metal ions.

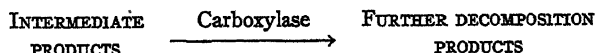
The picture of the combined vitamin and ion effect can best be presented by assuming that lactate, succinate, and fumarate must first be transformed into an oxidation product by reactions requiring the presence of metal ions rather than of thiamin. Following this, the further fate of the intermediate products would involve reactions in which thiamin (carboxylase) participates. The first phase in the decomposition of lactate, succinate, and fumarate would then be slow if the cells had been grown in a medium with an inadequate supply of minerals, but the rate of this oxidation should be increased by the addition of the necessary cations alone. Only when the rate of formation of intermediate substances exceeds the capacity of the carboxylase system present would the addition of thiamin have a stimulatory effect.

If, on the other hand, the rate of production of intermediate substances were high, due to the presence of sufficient enzymatic capacity for the initial reaction, the primary oxidation products would tend to accumulate in the presence of a limiting carboxylase supply. A case in point is the well known fact that pyruvic acid accumulates during the metabolism of sugar or lactate by thiamin-deficient organisms and tissues. The addition of thiamin would then cause an increase in metabolic rate, due exclusively to an effect on the second phase. This scheme may be illustrated by the following diagram.

Phase 1. Limited by cation supply:



Phase 2. Limited by thiamin supply:



In those cases where the rate of the first phase, although not maximal due to a cation deficiency, is nevertheless greater than the capacity of the enzymes operative in the second phase, the addition of either cations or of thiamin alone would cause an increase in the rate of oxygen consumption. However, the increase in rate of oxygen consumption due to the addition of thiamin alone can only be due to an increased

capacity for carrying out the second phase. Since, in the case of lactic acid decomposition, the rate of the second phase of the reaction can be determined directly by a study of the metabolism of pyruvic acid, the idea here developed can be tested experimentally. Fortunately, the publication of Quastel and Webley contains all the necessary data for such a comparison. These data have been assembled in Table II.

It can be seen that the predictions agree with the actual measurements. When the rate of the first phase is not altered, the effect of the addition of thiamin is identical for the oxidation of both lactate and pyruvate. This is seen to be true at both a low and high rate of the first phase.

The same effects can be shown for succinic and fumaric acids, where oxaloacetic and pyruvic acids could be expected as intermediate products. The data for succinate and fumarate are recorded in Table III.

TABLE II

*Quastel and Webley's Data for the Oxidation of Pyruvic (PA) and Lactic (LA) Acids, Arranged to Show the Possibility of Interpreting the Oxidation of Lactic Acid to Occur in Two Phases—the Rate of the First Governed by the Presence of Cations (C), the Second by Thiamin (T)*

Addition to deficient cells	Reaction involving cations	Reaction involving thiamin	$Q_{O_2}$	Difference in $Q_{O_2}$ due to thiamin
PA .....	None	Slow	20	17
PA + C + T.....	None	Fast	37	
LA .....	Slow	Slow	35	17
LA + T.....	Slow	Fast	52	
LA + C.....	Fast	Slow	53	19
LA + C + T.....	Fast	Fast	72	

In the oxidation of succinic and fumaric acids by deficient cells the increase in rate of oxygen consumption caused by the addition of thiamin is not so high when cations are also present; *i.e.*, when phase 1 occurs at a rapid rate. A simple explanation for this discrepancy is based upon the assumption that the carboxylase system may now have become limiting. Although adequate to cope with the supply of intermediate products furnished by phase 1 when this proceeds slowly, it may not be supplemented sufficiently by thiamin addition to cause a commensurate decarboxylation of these products when phase 1 proceeds at a maximum rate. Here the comparison with the decomposition of pyruvic acid, involving a single decarboxylation, is not entirely justified because in the breakdown of succinic and fumaric acids *via* oxaloacetic acid two decarboxylation reactions occur.

As another possibility it may be assumed that the over-all metabolic rate in the presence of both cations and thiamin is actually limited by the capacity of the final oxygen-activating systems. A decision between these alternative hypotheses would rest upon the evaluation of the significance of the  $Q_{O_2}$  increments. The higher value

with fumarate favors the latter explanation, but the difference in increment for succinate and fumarate in the presence of cations is too small to make this a convincing argument.

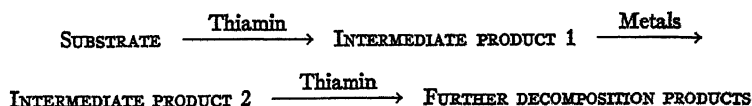
The stimulatory effect of thiamin and metal ions on the oxidation of acetate by Quastel and Webley's organism is different from that observed with lactate, pyruvate, succinate, and fumarate. In the case of the oxidation of this fatty acid, neither the cations nor thiamin alone permit the remarkable stimulation obtained when both are added together. This situation may be explained by assuming that a preliminary reaction, in which vitamin B<sub>1</sub> is involved, is required prior to the formation of a

TABLE III

*Quastel and Webley's Data for the Oxidation of Pyruvic (PA), Succinic (SA), and Fumaric (FA) Acids, Arranged to Show the Possibility of Interpreting the Oxidation of Succinic and Fumaric Acid to Occur in Two Phases—the Rate of the First Governed by the Presence of Cations (C), the Second by Thiamin (T)*

Addition to deficient cells	Reaction involving cations	Reaction involving thiamin	Q <sub>O<sub>2</sub></sub>	Difference in Q <sub>O<sub>2</sub></sub> due to thiamin
PA .....	None	Slow	20	17
PA + C + T .....	None	Fast	37	
SA .....	Slow	Slow	30	19
SA + T .....	Slow	Fast	49	
SA + C .....	Fast	Slow	58	9
SA + C + T .....	Fast	Fast	67	
FA .....	Slow	Slow	28	17
FA + T .....	Slow	Fast	45	
FA + C .....	Fast	Slow	53	12
FA + C + T .....	Fast	Fast	65	

compound which can undergo dehydrogenation, a reaction for which metal ions are needed. The reactions involved in the oxidation of acetic acid may be represented by a series of steps.



It is improbable that such a preliminary step would involve a decarboxylation of acetic acid because biochemical decarboxylations have been observed exclusively with keto acids. Since carboxylase is likely to function in the carboxylation of acids as well as in their decarboxylation, the preliminary step involved in the oxidation of acetate by these bacteria might be the linking together of two acetate molecules to form acetoacetate. This reaction may be considered as equivalent to a carboxy-

lation and hence might require carboxylase (*i.e.* thiamin). The consumption of oxygen in the decomposition of acetate by this scheme, should, therefore, be preceded by a step in which thiamin functions. The oxidation proper would involve the cooperation of the cation-requiring systems. In such a manner the observed effect of thiamin and cations on acetate oxidation by Quastel and Webley's organism could be readily harmonized with the concept that here, too, thiamin functions only as a building block of carboxylase.

*Theoretical Considerations on the Rôle of Thiamin in the Metabolism of Prototheca zopfii*

The experiments demonstrating a thiamin effect on the oxidation of acetate by *Prototheca zopfii* cannot as yet be interpreted in so detailed a manner. So far as is known, this alga has never been cultured in mineral-deficient media and, therefore, it is impossible to interpret the available data on the oxidation of acetate in the light of specific stepwise reactions. Whether thiamin has an *initial* or a *secondary* effect on the oxidation of this simple fatty acid by *Prototheca* must be left out of consideration for the present. At any rate, the function of thiamin in the oxidation of acetate need not be considered to be other than as a building block of carboxylase.

Krebs and Eggleston (21) have presented the hypothesis that the principal function of carboxylase in animal tissue, and also in certain higher plants, molds, and bacteria, is not directly concerned with the oxidation of pyruvate but with the preparatory reaction. The reaction, for which only indirect evidence has been presented, is a carboxylation in which oxaloacetic acid is synthesized from pyruvic acid and carbon dioxide. The oxaloacetic acid so formed is then used in the 4-carbon-dicarboxylic acid cycle of hydrogen-transporting substances known as the "Szent-Györgyi cycle." It also forms the basis of the citric acid cycle postulated by Krebs as an integral part of oxidative metabolism. Smyth (22) has presented supporting evidence for such a mode of oxidation of pyruvic acid in the case of *Staphylococcus aureus*. Using vitamin-deficient cells, Smyth found that thiamin could be replaced by the addition of a 4-carbon-dicarboxylic acid. That is, the addition of either thiamin or a compound such as oxaloacetic acid increased the rate of oxidation of pyruvic acid by vitamin-deficient cells of the staphylococcus.

Although Krebs' hypothesis for the mechanism of pyruvic acid oxidation is most attractive, its general occurrence has not been well substantiated as yet. It is to be remembered that this scheme would hardly fit in with the convincingly established fact that the enzyme carboxylase itself causes the quantitative decarboxylation of pyruvic acid. If thiamin is postulated to act principally as an enzyme system for the synthesis of oxaloacetic acid, the importance of the decarboxylation mechanism recedes entirely into the background. It is true that the mechanism for the oxidative degradation of pyruvic acid, pro-

posed by Krebs in connection with the "citric acid cycle," would make a decarboxylation unnecessary. However, there are entirely too many facts, supporting the view that decarboxylations, too, are of primary importance in metabolism, to permit the discard of this mechanism.

In view of the significant position pyruvic acid holds as an intermediate product in the metabolic processes in general, experiments were conducted to determine whether the findings of Smyth on the substitution for thiamin of members of the 4-carbon-dicarboxylic acid are applicable in the case of *Prototheca*. The results of experiments in which succinic, fumaric, or malic acids were added in catalytic amounts to suspensions of vitamin-deficient cells oxidizing pyruvic acid do not support Krebs' postulate. In no case did the addition of these acids result in stimulation of the rate of oxygen utilization. The presence of thiamin seems essential to permit the oxidation of pyruvic acid by *Prototheca zopfii*. This observation tends to throw additional doubt on the general validity of the citric acid cycle as the only mechanism for the oxidative decomposition of pyruvic acid.

The results obtained with *Prototheca*, therefore, lead to the conclusion that the function of thiamin as a building block for carboxylase implies the regular occurrence of genuine decarboxylation reactions. Whether these must be considered as straight decarboxylations or as oxidative decarboxylations remains for further investigation. In the event that an oxidative decarboxylation is involved in the metabolism of *Prototheca*, the combined function of carboxylase and an additional hydrogen acceptor would, of course, be indicated (see, e.g., Long and Peters (23) and Peters (24)). However, the main argument put forward here to stress the importance of thiamin (carboxylase) as a decarboxylating agent, remains unaltered. The experimental results definitely indicate that the need of thiamin by *Prototheca* is immediately concerned with the decomposition of  $\alpha$ -keto acids.

#### STUDIES ON THE OXIDATION OF GLYCOLIC ACID BY PROTOTHECA ZOPFII

Barker's investigation of the mechanism involved in the synthesis of carbohydrate from simple fatty acids, such as acetic, by *Prototheca* resulted in a deadlock because any scheme that could be postulated for the oxidative degradation of acetate involved acids as intermediate products which the organism was incapable of utilizing as substrates. When the alga was found capable of oxidizing such acids, and conditions could be specified under which a study of their metabolism is possible, an experimental reexamination of conceivable mechanisms for the decomposition of simple fatty acids became feasible.

An investigation of the mechanism involved in the oxidative assimilation of a utilizable substrate must of necessity consider two important phases of the process—the decomposition of the substrate and the synthesis of cell material.

The theoretical basis upon which the subsequent experiments were conducted will be developed in the following section.

### *Theoretical Considerations*

Although it has long been known that a definite relationship exists between the breakdown and assimilation of foodstuffs, oxidation and synthesis have been considered as more or less separate reactions in which the assimilation reactions, giving rise to products possessing greater free energy than that of the substrate, can acquire energy from the simultaneously occurring dissimilation process in which the free energy decreases. As long as the amount of energy liberated in the catabolic process is in excess of that required by the anabolic reactions, energetically coupled reactions of this type are quite possible on the basis of thermodynamic considerations.

A clearer understanding of the mechanism involved in biochemical processes concerned with the breakdown of foodstuffs has resulted in the possibility of also interpreting assimilatory processes as chemically intelligible step-reactions, not materially different from those operative in catabolism. The general principles of the mechanisms involved in catabolic processes may be briefly summarized. Such a biochemical process can be considered to consist of a chain of individual step-reactions, each step constituting a simple, chemically understandable type of reaction whose common property is the transference of hydrogen from one constituent to another. Each step is a thermodynamically exergonic reaction.<sup>2</sup> This attempt to interpret anabolic and catabolic processes as being closely intermingled chemical reactions does not deny the existence of energetic relations but aims at elucidating the chemical mechanisms for energy transfer which would obviate the necessity of considering the energetic coupling of two sets of chemical reactions which are not known to possess any material link.

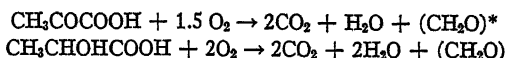
The first broad attempts to formulate a chemical mechanism of energy transfer were those of Kluver (26, 27) who opened the way for further advances. Although the proposed "mechanisms" may no longer be considered as tenable or probable, the fundamental principle remains a valuable working hypothesis. This embodies the idea that, in the course of the degradation of the substrate, intermediate products arise which can be exergonically converted into the products of assimilation. Thus the structure of the intermediate products is all important in the process of synthesis while the gradual degradation of the initial substrate down to the point of the formation of the essential product plays no *direct* rôle in the assimilation process itself, but is merely the preparation of the essential building block for synthesis. Giesberger (3) developed this idea in his concept of "chip-respiration" in which carbon dioxide and water formed during the oxidative process are regarded as "chips" or waste products of the main reaction. This concept of oxidative assimilation, therefore, places the main emphasis upon the structure of the raw material. Any compound which could be postulated to give rise to an intermediate product possessing the characteristics required to enable it to serve as an initial substrate for exergonic synthetic reactions, would serve the purpose of synthesis to the extent to which it

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<sup>2</sup> The term "exergonic" is used in preference to "exothermic" in accordance with Coryell's proposal (25).

could contribute the necessary intermediates. Although little work has been done on a study of the mechanisms of assimilation there are indications in the literature which lend strong support to the above views.

Clifton and Logan (28), investigating the oxidative assimilation of various compounds by *Escherichia coli*, found that the oxidation of lactic acid proceeds in a manner similar to that of pyruvic acid. The oxidation of these two compounds can be represented by the following equations:



\* $(\text{CH}_2\text{O})$  is here used to represent a compound having the empirical formula of a carbohydrate.

It is a general observation that, in the course of its oxidation, lactate passes through the stage of pyruvate by the loss of two atoms of hydrogen. Therefore, it may be assumed that the same intermediate products would arise in the oxidation of each of these two acids. This is substantiated by the above equations which show that *Escherichia coli* converts one-third of the total carbon of both lactate and pyruvate into primary assimilation products, despite the fact that the concomitant oxygen consumption is materially greater with lactate than with pyruvate. This implies that simple energetic considerations fail to account satisfactorily for the situation because more energy becomes available in the oxidation of lactate than in the oxidation of pyruvate. Conversely, the formation of carbohydrate storage products from pyruvate would require more energy than from lactate. If, therefore, catabolic and anabolic processes represented merely two types of reactions, coupled energetically, then the extent of assimilation should be appreciably greater with lactate than with pyruvate. The very fact that equal portions of both substrates appear in the form of assimilation products thus strongly supports the contention that the nature of special intermediate products is far more important than energetic relationships.

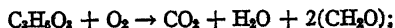
Clifton and Logan also found that the same amount of assimilation occurs during the oxidation of succinic as during the oxidation of fumaric acid, although the free energy of succinate is greater than that of fumarate.

The results of Doudoroff's (29) studies on the oxidation of various substances by *Pseudomonas saccharophila* have shown that this organism carries out an oxidative assimilation in much the same manner as that described by Barker (2), Giesberger (3), and Clifton and Logan (28) for other microorganisms. The broader studies of Doudoroff showed that sugars, both hexoses and disaccharides, as well as lactic and pyruvic acids are respired with the complete oxidation of one-third of the substrate and the assimilation of two-thirds. The reaction for each substrate could be represented by a simple stoichiometric relationship, as shown by the following equations:

Pyruvic acid

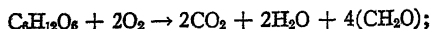


Lactic acid





## Glucose



## Sucrose



From these equations it is apparent that the thermodynamic efficiency with which these substances are assimilated increases in the order carbohydrate, lactate, pyruvate, and that the actual extent of synthesis is directly dependent upon the number of carbon atoms contained in each. Here again, a comparison of the energy released in the oxidation of carbohydrates, pyruvate, and lactate indicates that synthesis is consequently dependent on a chemical mechanism concerned with the intermediate products of metabolism rather than on a purely energetic coupling between separate reactions of oxidation and of synthesis.

By the demonstration that pyruvic acid could be isolated as an intermediate product in the oxidation of glucose, Doudoroff was able to support the indications, presented in the equations for the oxidative assimilation of the sugars, that the metabolism of the mono- and disaccharides would proceed by way of the three-carbon compounds.

*Lactate and Pyruvate Oxidation by Prototheca zopfii*

The above examples clearly show that a comparison of the decomposition of structurally related compounds has, in the case of *Escherichia coli* and *Pseudomonas saccharophila*, supported the idea that a chemical mechanism is operative in processes of oxidative assimilation. Since previous experiments on the oxidative metabolism of *Prototheca zopfii* had not included such structurally related compounds, a comparative study was made of the oxidation of pyruvic and lactic acids by this colorless alga.

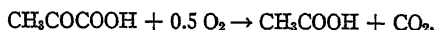
The oxidation of these two acids by *Prototheca* was found to correspond to that for *Escherichia coli* and to differ markedly from that for *Pseudomonas saccharophila*.

During the rapid oxidation of pyruvic acid by *Prototheca*, the ratio of carbon dioxide production to oxygen consumption was found to be between 1.20 and 1.37 with an average of 1.31 for four experiments. The theoretical value required by the equation is 1.33. The R.Q. decreased gradually to that found for autorepiration at the time when the acid was completely used up. Data from a number of experiments indicate that autorepiration is completely suppressed during the oxidation of pyruvic acid. The R.Q. for lactate oxidation was found to be approximately 1.0, which also agrees with the theoretical.

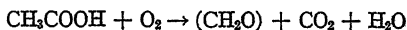
Again, the above experimental results fit in much better with a chemical than with a strictly energetic concept of the mechanism for an assimilatory process. The equations show that the conversion of lactate to pyruvate may well proceed without being accompanied by the formation of reserve materials. It is even possible to postulate further a rational pathway for the subsequent

decomposition of pyruvic acid which is entirely in harmony with the experimental results.

The experiments on the influence of thiamin on pyruvic acid decomposition by *Prototheca zopfii* make it logical to accept the occurrence of a decarboxylation mechanism in the oxidation of pyruvic acid. In view of the complete lack of alcohol production by *Prototheca* under anaerobic conditions, the most likely fate of pyruvic acid would appear to be an oxidative decarboxylation. This would result in the production of equimolar amounts of carbon dioxide and acetic acid and would involve the utilization of one-half mol of oxygen per mol of pyruvic acid:

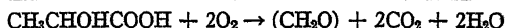
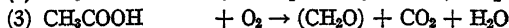
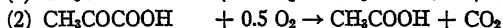
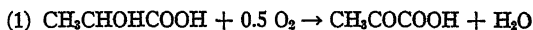


The oxidation of acetic acid has been extensively investigated by Barker, and can be expressed by the equation:



The sequence of stages in the oxidation of pyruvic acid could then be represented by a summation of these two equations. This yields a final equation identical with that experimentally determined.

Similarly the consecutive steps for the oxidation of lactic acid could be formulated as follows:



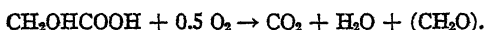
Again, the final equation is in complete agreement with the one derived from experimental data. The first two steps are simple reactions for which much evidence had been accumulated in a number of instances and with a variety of organisms. These steps may be considered as elementary ones whose intimate mechanism can be investigated only by special enzyme studies. This is, however, not true for the third state, which not only leaves the question of intermediate products in the acetate oxidation unanswered, but which also "hides" the mechanism of the assimilation process proper. It is, therefore, apparent that the reasons which prompted the postulation of these series of reactions for the decomposition of pyruvic and lactic acids inevitably led to the desire to study the mechanism of acetate oxidation in more detail.

#### *Experiments on the Oxidation of Glycolic Acid by Prototheca zopfii*

Of the two main pathways for the decomposition of acetic acid outlined in the introduction the one involving the successive oxidation to glycolic and glyoxylic acids appears to be the simpler. If the oxidative metabolism of

acetic acid were to proceed through glycolic acid as an intermediate, an investigation of the oxidation of glycolic acid could be expected to show that its decomposition occurs in a manner similar to that described for acetic acid, and an oxidative assimilation as a result of the decomposition of glycolic acid could be expected which would be even more spectacular than that found in the case of acetic acid. The formation of assimilation products from such simple compounds would involve only the methylene group, the  $\text{—COOH}$  group being lost. The former group is more oxidized in the case of glycolic acid than it is in acetic acid and therefore more nearly conforms to the empirical formula of the assimilated material. Therefore, the same proportion of assimilation might occur per mol of glycolic as per mol of acetic acid, while being accompanied by an oxygen consumption of only one-half the magnitude. This situation is similar to that discussed above in the comparison of the oxidation of lactic and pyruvic acids.

On the basis of simple stoichiometric relationships, the equation for glycolic acid might be represented as:



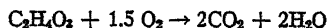
Since the energy obtainable from the oxidation of acetic acid is twice the amount obtainable from glycolic acid, a demonstration that the oxidative assimilation of glycolic acid could be represented by the above equation would effectively rule out the occurrence of coupled catabolic and anabolic reactions in a strictly energetic sense.

The addition of 0.01 mM of glycolic and 0.01 mM of glyoxylic acid to aliquot suspensions of non-proliferating cells of *Prototheca zopfii* resulted in an increase in the rate of oxygen consumption over that of control suspensions. The higher respiratory rates show *Prototheca* to be capable of utilizing the two acids postulated as occurring as intermediates in the Bernhauer scheme for the oxidation of acetic acid.

#### *Quantitative Studies on the Oxidation of Glycolic Acid*

Attempts to establish a balanced equation for the oxidation of glycolic acid by *Prototheca* resulted in the unexpected observation that the amount of oxygen consumed was far in excess of the amount of oxygen that would be required to bring about a *complete* combustion of this substrate.

The complete oxidation of 0.01 mM of glycolic acid, in agreement with the following equation:



requires the uptake of 336 mm.<sup>3</sup> of oxygen.

The addition of 0.01 mM of glycolic acid to suspensions of cells having a moderately high rate of autorespiration, resulted in the utilization of 1.89 times

the amount of oxygen needed for complete combustion. If the oxygen consumed is uncorrected for autorepiration, this value is increased to 2.44 times. The addition of 0.01 mm of glycolic acid to suspensions of cells having a lower rate of autorepiration resulted in a value of 1.54 times corrected for autorepiration and 1.85 times if no correction is made.

The addition of different amounts of glycolic acid to several equal portions of cell suspensions showed conclusively that in all cases the oxygen consumption attained values greatly in excess of those required for complete oxidation of the added substrate. Data from nine different experiments, in which suspensions of cells having quite different rates of autorepiration were used, show that the amount of oxygen consumed during the oxidation of equal amounts of glycolic acid varied from 1.34 to 3.57 times the amount of oxygen that would be required for complete oxidation of this substrate. Each of these values is that obtained after correction for autorepiration of control suspensions.

#### *Explanation of the Excess Oxygen Consumption in the Oxidation of Glycolic Acid*

The unexpected oxygen consumption upon the addition of glycolic acid to suspensions of non-proliferating cells of *Prototheca* indicates that this acid must exert a catalytic effect on the metabolism of this organism.

To test this hypothesis, aliquot portions of heavy suspensions of washed cells were suspended in phosphate buffer solution at pH 4.0. Each portion was placed in shallow layers in rotating bottles and incubated at 30°C. for 12 hours. One portion was given no added substrate but allowed to carry on endogenous respiration only, resulting in the production of "starved" cells. During the incubation period, small amounts of glycolic acid were added to the second portion at intervals of sufficient duration to insure that meanwhile the previous addition had been completely oxidized.

The autorepiration of the normal "starved" and the glycolic acid-treated organisms was measured at the end of the incubation period. The rate of respiration of the organisms which had previously been oxidizing glycolic acid was found to be but 72.2 per cent of the rate of the "starved" cells. This observation indicates that the reserve cell material had been oxidized more rapidly in the presence of glycolic acid than in its absence, and that the residue remaining available for autorepiration was sharply reduced in the experimental organisms.

Addition of 0.001 mm of glycolic acid to 2.0 ml. suspensions of both types of cells resulted in a rapid uptake of oxygen. The cells previously treated with glycolic acid consumed a volume of oxygen, over that for autorepiration, equal to 1.75 times the amount needed for complete combustion of the acid added. In contrast, the normally starved cells utilized an amount of oxygen equal to 2.47 times that needed for complete oxidation. This may also be

taken as an indication that the oxidation of glycolic acid by non-growing cells of *Prototheca* causes the oxidation of cell material in addition.

In two experiments carried out with cells having a very high rate of autorespiration the addition of 0.001 mM of glycolic acid resulted in the uptake of an amount of oxygen, corrected for autorespiration, equal to 3.57 and 3.22 times the amount necessary for total combustion of the acid. This may be compared with an average of 1.92 for four experiments carried out with cells having a more "normal" rate of autorespiration.

That the oxidation of glycolic acid by *Prototheca* does affect the autorespiration is further evidenced by the results obtained in experiments in which two successive additions of 0.001 mM of glycolic acid were made to suspensions of cells having a high rate of autorespiration. The first addition resulted in the uptake of a quantity of oxygen equal to 3.16 times the amount needed for complete oxidation of the acid. An equal amount of the acid added to the suspension at the time the first portion was completely decomposed caused an uptake of oxygen equal to but 2.08 times that necessary for total oxidation. The addition of 0.002 mM of glycolic acid to the suspensions resulted in the uptake of the same amount of oxygen as when the substrate was added in two equal portions.

To determine whether glycolic acid has an effect on the ability of the cells to carry out an oxidative assimilation, and also to test if glycolic acid could cause an oxidation of newly assimilated cell materials, experiments were carried out in which the oxygen consumption of aliquot suspensions of cells treated in four different ways, was compared. To one sample 0.001 mM of glycolic acid alone was added; another, initially treated in the same way, was supplied with 0.01 mM of acetate after the glycolate had been consumed; both acids, in the above stated amounts, were added simultaneously to the third portion; and in the fourth suspension the glycolate was introduced following the decomposition of an initial supply of 0.01 mM of acetate. The data so obtained are presented in Fig. 6.

The addition of glycolic acid alone to the suspensions of cells used for these experiments caused an oxygen uptake of 1.34 times the amount required for the complete oxidation of the acid. The subsequent addition of acetic acid resulted in the consumption of 1 mol of oxygen per mol of acetate which is identical with the values obtained for the oxidative assimilation of this substrate by "normal" cells. The suspension to which both acids were simultaneously added, showed a total oxygen uptake of the same amount as the total consumed by the suspension to which glycolic acid was added prior to the addition of acetic acid. The results of these two experiments indicate that glycolic acid has no effect on the ability of *Prototheca* to assimilate a typical substrate. In the case in which glycolic acid was added after the suspension had completed its oxidative assimilation of acetic acid alone, the addition of

0.001 mM of glycolic acid resulted in the uptake of 1.64 times the amount of oxygen required for the complete oxidation of the glycolic acid. A comparison of this value with that obtained for the oxidation of glycolic acid by an aliquot portion of cells which had not oxidized acetic acid (1.34) indicates that glycolic acid caused the oxidation of some of the cell material assimilated during the oxidation of acetic acid and may be considered as additional evidence that the

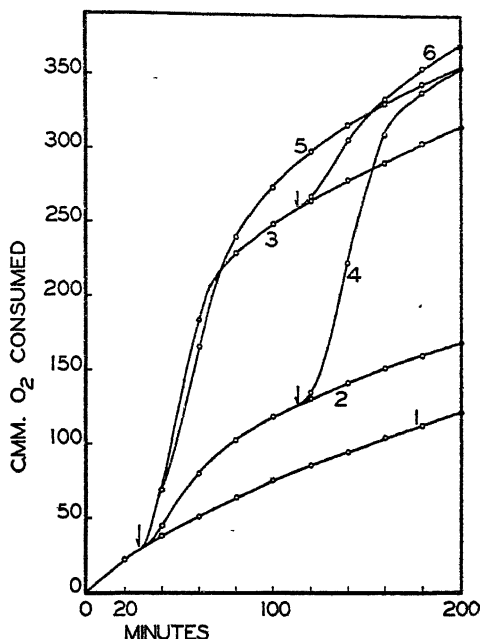


FIG. 6. Influence of glycolic acid on the oxidative assimilation of *Prototheca zopfii*: (1) autorespiration; (2) 0.001 mM glycolic acid; (3) 0.01 mM acetic acid; (4) 0.01 mM acetic acid added after decomposition of 0.001 mM glycolic acid; (5) glycolic acid and acetic acid added simultaneously; (6) 0.001 mM glycolic acid added after decomposition of 0.01 mM acetic acid. Arrows indicate time at which substrates were added.

oxidation of glycolic acid by *Prototheca* has an effect on the autorespiration of these cells.

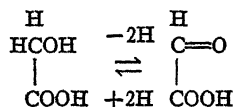
#### *Comparison of the Action of Glycolic Acid with That of Known Biochemical Catalysts*

With the exception of glycolic acid, all the substrates so far tested with *Prototheca zopfii* are oxidized in such a manner that a simple stoichiometric relationship exists between the number of substrate molecules disappearing, and the number of molecules of oxygen consumed and of carbon dioxide pro-

duced. These relationships permit the formulation of simple, balanced equations, representing the over-all result of the metabolic activity. Also, in all cases, with the exception of glycolic acid oxidation, the quantity of oxygen consumed appears to be a definite fraction of that required for complete oxidation of the substrate.

The unexpectedly high values obtained for oxygen consumption during the oxidation of glycolic acid can be explained only on the basis that this substance functions as a "respiratory catalyst." Thus it becomes attractive to compare the action of glycolic acid with that of substances known to be biochemical catalysts.

Since the general effects of glycolic acid on the oxidative metabolism of *Prototheca zopfii* are so very similar to those which led to the proposal of the Szent-Györgyi (30-32) and Krebs cycles (33, 34) the question arises whether it is possible that glycolic acid also might participate in a similar catalytic cycle. The two previously postulated cycles depend largely upon the occurrence of related hydroxy and keto acids. Such relations are readily conceivable for the system glycolic-glyoxylic acids:



Therefore, the possibility of a glycolic acid-glyoxylic acid cycle, functioning as a simple type of hydrogen-transporting system, is far from remote.

Against this interpretation is, however, the fact that glyoxylic acid is respired in a "normal" manner by *Prototheca*. This substrate does not appear to possess any catalytic properties such as it would demonstrate if it were a participant in a cyclic reaction.

While this observation makes it, therefore, unlikely that the catalytic effect of glycolic acid on cellular respiration by *Prototheca* is due to its action as a factor of a simple hydrogen-transporting system, a more complicated manner of its participation in metabolism may be considered. The function of glycolic acid in a mechanism similar to that postulated for oxaloacetic acid in the Krebs cycle would imply that glycolic acid is condensed with some oxidizable substance resulting in the synthesis of a compound which is more easily oxidizable than the metabolite itself. The nature of this type of mechanism as applied to glycolic acid is not clear. It is theoretically possible that two mols of glycolic acid could couple to form malic acid in a manner similar to the postulated synthesis of succinic acid from two mols of acetic acid in the Thunberg scheme. However, the addition of malic acid to suspensions of non-proliferating cells of *Prototheca* does not produce a catalytic effect as would be expected if this substance were to arise from glycolic acid "condensation."

The observed action of glycolic acid does suggest an interplay of this substance with some oxidizable cell constituent. However, the nature of the latter is completely unknown and therefore further speculation at this time would seem futile.

It is impossible to evaluate the available quantitative data to determine the relation between glycolic acid used, oxygen consumed, and carbon dioxide produced because it is impossible to separate the gaseous exchange due to the oxidation of glycolate from that arising as a result of the induced oxidation of cell material. For a detailed discussion of the general difficulties encountered in evaluating the portion of metabolism to be ascribed to autorespiration, reference is made to the work of Barker (2), Doudoroff (29), and Thomas (35).

All that can be definitely concluded from the present studies on the oxidation of glycolic acid is that this substance acts in a catalytic capacity and, therefore, the mechanism of acetate oxidation does not go through glycolic acid as an intermediate.

The discovery that *Prototheca* can utilize substituted acids has made possible an investigation of the intermediate stages of acetate oxidation. However, the first approach based on Bernhauer's scheme for acetate degradation, yielded information which makes it necessary to discard this as a likely pathway. With the mechanisms proposed for acetate breakdown thus restricted, studies on other possible intermediates are necessary. Preliminary investigations of one of the condensation reactions of acetate, that of Thunberg, have yielded certain results which indicate this scheme also to be unlikely. By elimination, therefore, the most profitable mechanism for acetate degradation remaining for future investigation is that of a condensation reaction with the formation of acetoacetate and its subsequent oxidation.

#### SUMMARY

The metabolism of *Prototheca zopfii* was investigated in an attempt to establish the specific function of its growth factor, thiamin. A study of the oxidative decomposition of various substrates by this organism demonstrated that the addition of catalytic amounts of thiamin to vitamin-deficient cells causes a pronounced stimulation in the rate of oxygen utilization during the degradation of certain compounds.

The phosphoric ester of thiamin is known to be the prosthetic group of carboxylase. The fact that this enzyme is involved in the decomposition of pyruvic acid suggested that this  $\alpha$ -keto acid might be an important intermediate product in the metabolism of *Prototheca*. Pyruvic acid, however, was not included in the list of organic substances which Barker had reported as utilized by this alga. Barker's observations were confirmed, but subsequent experiments led to serious doubts as to the validity of his interpretation. Further investigations resulted in the establishment of environmental condi-



tions which permit this alga to readily decompose pyruvic acid, as well as nearly all other organic acids tested. This can be accomplished by providing a milieu of sufficiently low pH to insure the presence of undissociated acid molecules.

The stimulatory effect on the rate of oxygen consumption, caused by the addition of minute amounts of thiamin to suspensions of vitamin-deficient cells of *Prototheca* respiring pyruvic acid, indicates that the presence of thiamin results in the synthesis of enzyme systems which are involved in the decomposition of pyruvic acid.

Experimental data on the oxidation of pyruvic acid and other organic compounds are discussed in the light of various hypotheses which have been advanced concerning the rôle of carboxylase in the decomposition of pyruvic acid. The conservative conclusion which can be drawn from the available information is that there appears to be no justification for a belief that thiamin and carboxylase are functional in biochemical reactions other than in decarboxylation and carboxylation processes.

The discovery of the ability of *Prototheca* to utilize substituted and dicarboxylic acids led to further studies on the mechanism of oxidative assimilation. The results of these investigations are in agreement with those of Clifton and Logan, and of Doudoroff, and indicate the existence of a relatively simple chemical mechanism of assimilation rather than of a strictly energetic coupling of catabolic and anabolic reactions.

A consideration of possible mechanisms for the oxidative assimilation of pyruvic and lactic acids indicates acetic acid as the most likely starting point for the assimilatory process proper.

Experimental investigations of the mode of acetate breakdown began with studies on the oxidation of glycolic acid. This substance is shown to be an oxidation catalyst in the metabolism of *Prototheca zopfii*. The exact nature of the catalytic function has not yet been determined.

It is a great satisfaction to be able to thank Dr. C. B. van Niel for suggesting the present study and for the stimulating advice, the sagacious criticism, and the encouragement which he gave so unstintingly during the time it was my privilege to work under his inspiring direction. In addition, I am very much indebted to my associates and to various members of the staff of the Hopkins Marine Station for their numerous courtesies and willing assistance at all times.

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# THE QUANTUM YIELD OF OXYGEN PRODUCTION BY CHLOROPLASTS SUSPENDED IN SOLUTIONS CONTAINING FERRIC OXALATE\*

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## INTRODUCTION

The evolution of oxygen from chloroplasts suspended in solutions containing ferric oxalate was discovered by Hill (1937) and has aroused considerable interest because this reaction appears to be similar in many ways to the oxygen evolution step of normal photosynthesis. It has been investigated further by Hill (1939), Hill and Scarisbrick (1940 *a*, 1940 *b*, 1940 *c*), and by French and Anson (1945), and has been discussed by Johnston and Myers (1943). With the intent of further characterizing the nature of this reaction, we have made some measurements of its quantum yield. If the quantum yield of the Hill reaction was approximately the same as that for oxygen production by normal photosynthesis, it might be taken as an indication that the two processes were similar or possibly identical. The importance of knowing whether the Hill reaction is closely related to the oxygen evolution step of photosynthesis lies in the fact that the Hill reaction takes place in material more amenable to biochemical investigation (*i.e.* isolated, dried, or disintegrated chloroplasts (French and Anson 1945)).

The quantum yield of photosynthesis in intact *Chlorella* cells has been found to be about 0.09 molecule of oxygen per quantum of light energy by Emerson and Lewis (1943), by Manning, Stauffer, Duggar, and Daniels (1939), and by Rieke (1941). In contrast to this, Gaffron (1927) has reported values of about 1.0 for the quantum yield of photooxidation of organic substrates by chlorophyll in solution. Very small yields of about 0.01 have been postulated for photooxidation processes in live plants by Franck and French (1941).

## EXPERIMENTAL PROCEDURES

1. *Preparation and Treatment of Suspensions.*—A large number of the experiments were performed with chloroplast suspensions obtained from market spinach. The healthy young leaves were placed in tap water cooled with ice cubes and were exposed to the illumination from a north window for an hour before use. The leaves were drained free of water and were macerated in a Waring blender which contained

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enough 0.5 M sucrose solution to cover the leaves. The liquid was filtered through a clean handkerchief and the chloroplasts collected by centrifugation as described by French and Anson (1945). All operations were carried out in a refrigerator room at a temperature of about 5°C. The desired volume of chloroplast suspension was added to a solution which contained 0.05 M  $K_2C_2O_4$ , 0.01 M  $FeNH_4(SO_4)$ , 0.02 M  $K_3Fe(CN)_6$ , 0.20 M sucrose, and 0.17 M sodium sorbitol borate.<sup>1</sup>

The solution containing the experimental material was placed in a round, flat-bottom manometric vessel with one side arm. The side arm was filled with 0.5 ml. of a 10 per cent NaOH solution which was prevented from "creeping" with a paraffin barrier. To shorten the temperature equilibration time after the chloroplast suspension was added to the reagents, it was customary to first bring the vessel and the reagents to the temperature of the thermostat, and then add the chloroplast suspension to the reagents. When properly equilibrated, neither the reagents alone in dark or light, nor the reagents plus the chloroplast suspension in the dark, showed any pressure change after the manometer stopcocks were closed. Before the addition of the chloroplast suspension to the manometric vessel, chloroplast chlorophyll concentration was measured photometrically as described by French and Anson (1945). Preliminary experiments made with single Warburg manometers showed that long observation periods were necessary because of the small reaction rate. Construction of a differential manometer with a small bore capillary tube increased the sensitivity of the apparatus so that the readings could be made in much less time, with greater accuracy than with single manometers. This was imperative since it was found that at 10° C. and in contact with the Hill reagents, the chloroplast suspension lost a small part of its oxygen-producing ability during the first 5 minutes of the experiment. For this reason, in the calculation of the reaction rate, greater emphasis was given to the rate during the initial portion of the illumination period. A second illumination period, separated from the first by either a period of darkness or a period of white light illumination, was accompanied by a further decreased reaction rate. A protocol of the illumination procedure is shown in Fig. 1.

2. *The Differential Manometer.*—Since the differential manometer used in this work is slightly different from the original differential manometer described by Barcroft (1908), a brief description will be given here. The differential manometer used by Warburg and Negelein (1922), Emerson and Lewis (1943), and Rieke (1939) is built with the arms of the capillary some distance apart thus requiring a double cathetometer for precise reading of the level of the manometric fluid in either capillary. By construction of a manometer so that both capillaries were vertical and adjacent to each other as shown in Fig. 2, we have been able to measure the level difference directly with a Bausch and Lomb binocular dissecting microscope fitted with an ocular scale divided into 100 parts. Only one eyepiece was used. The microscope was mounted horizontally on a vertical rack and pinion held on an optical bench. The rack and pinion was movable through an angle of about 10° in a vertical bearing, its position at the two extremes being determined by adjustable screw clamps, so that at either setting one of the capillaries is in the center of the ocular scale. It was

<sup>1</sup>Stock sorbitol borate solutions of pH 6.1, 6.5, and 6.8 were used as a buffer as explained in Table III.

customary to place the image of the right capillary at the zero line of the ocular scale by vertical adjustment of the rack and pinion and then to shift the eyepiece to point at the left capillary, the difference in capillary height being read off the scale directly. Each scale division was equal to 0.114 mm. and the readings were reproducible to better than one division. The illumination of the manometer menisci was accomplished by a pen-type flashlight bulb with a built-in lens which was mounted on a separate rack and pinion. Readings were made while the manometer was shaking. The total height difference measurable in this way was about 1 cm. For greater

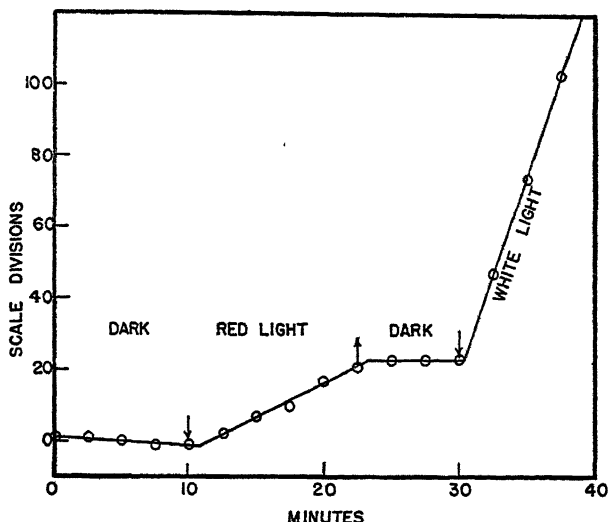


FIG. 1. The net pressure changes are plotted against time for Experiment 5 of Table III. The quantum yield is determined from the difference in slope between the red light period and an average of the two dark periods. The exposure to white light was made to be sure that the chloroplasts were still active after the experiment. The absence of a pressure change due to respiration of the chloroplasts during the dark period is caused by the fact that both the control and the experimental vessels contained similar material. This respiration is very small anyway as compared with the gas exchange due to the Hill reaction.

differences, the stopcock joining the upper part of the two capillaries was opened momentarily to equalize the pressure and to allow the new zero point to be established without opening either vessel to the air. Shaking the manometer and its vessels was accomplished by a rocking motion of 190 excursions per minute through a total path of 0.8 cm. Readings were made at 2.5 minute intervals. The cross-section area of the capillary was  $0.0525 \text{ mm}^2$ , somewhat too small for convenience since the viscosity of the fluid can cause a time lag in the equilibrium level with such a small capillary. A manometer fluid with a low vapor pressure, low viscosity, low density, low surface tension, and good wetting properties was found to be xylene containing about 2 per cent each of oleic acid, sorbitol trioleate (Atlas), and "modified" sorbitan trioleate (Atlas). No effort was made to find the precise optimum concentration

of these substances. The specific gravity of the solution used was 0.862 ( $P_0 = 11,980$  mm.). It can only be used in an all-glass system. Grease is readily dissolved by this solution.

3. *Light Production and Light Intensity Measurement.*—Fig. 3 is a diagram of the equipment used in this study. A 1000 watt T20 tungsten projection bulb with a C13 filament was used as a source and was run at 8.0 amp. instead of the rated 8.7 amp. in order to prolong its life. A 1 kw. voltage-regulating transformer supplied the current. A parabolic mirror with a diameter of 9 inches and a focus of 7 inches concentrated the light into the end of a Pyrex glass tube 2.5 inches in diameter and 3

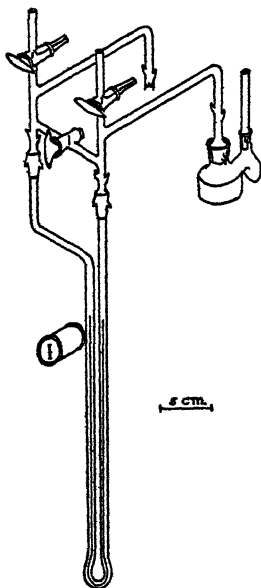


FIG. 2. The differential manometer. A description of its construction and use is found in the text.

meters in length. This tube was filled with distilled water and served to absorb most of the infrared light. After the light emerged from this tube it passed through a Jena RG5 filter and was concentrated on the vessel by means of two condenser lenses and a mirror. A diaphragm with a 1 inch circular hole was placed between the mirror and the manometric vessel. The mirror, diaphragm, and the vessel were placed in a water thermostat at a temperature of about  $10^{\circ}$  C. and constant to  $\pm 0.005^{\circ}$  C. as measured with a Beckmann thermometer. The rate of the reaction was shown to be directly proportional to the intensity of the light used in this work by the introduction of calibrated screens in the beam to reduce the intensity by a known amount. Quantum yields were not increased by this procedure so it was concluded that light was the limiting factor (Experiments 2, 12, 28, and 29 of Table III). Further evidence that the light was not too bright for the attainment of maximum efficiency at least for *Chlorella* photosynthesis is shown in Fig. 4 where a linear rela-

tionship is shown between the total incident energy multiplied by the fraction absorbed and the rate of photosynthesis.

The approximate wavelength distribution of this light beam is given in Table I. This was confirmed by visual observation with a spectroscope. The amount of stray radiation of wavelength longer than  $720\text{ m}\mu$  (about 6.8 per cent) was determined with the thermopile by interposing a Wratten filter, No. 88 in the light beam.

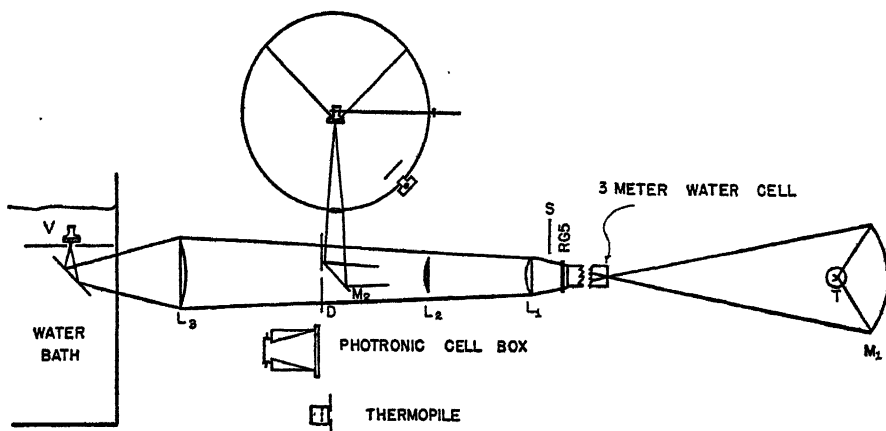


FIG. 3. A diagram of the equipment used for the production and measurement of the light used in this study. The photonic cell box is placed behind the diaphragm  $D$ , for calibration, and then in the water in place of the vessel  $V$ , to measure the total light incident on the vessel. The thermopile is later placed so that its sensitive surface is in the plane previously occupied by the diaphragm  $D$ , to measure the intensity of light used for the photonic cell calibration.  $T$ , tungsten bulb.  $M_1$ , parabolic mirror, focus = 4.5 inches, diameter = 10 inches.  $L_1$ , lens, focus = 8 inches, diameter = 4.5 inches.  $L_2$ , lens, focus = 7.5 inches, diameter = 4.5 inches.  $L_2$  is used only for calibration, in order that the light beam may be concentrated on the diaphragm,  $D$ . This lens and the diaphragm are removed during exposure of the chloroplasts in vessel,  $V$ , to the light beam.  $M_2$ , mirror, used only when vessel,  $V$ , is placed in the sphere for transmission measurements.  $L_3$ , lens, focus = 10 inches, diameter = 6.5 inches.  $S$ , shutter.

For the later experiments the amount of stray radiation was reduced by a further improvement of the light source. A band of light from  $660$  to  $695\text{ m}\mu$  was isolated from a tungsten lamp with a strip filament which was run at 70 amp. and 5 volts. Isolation was accomplished with the aid of a monochromator made of a  $4 \times 6$  inch transmission replica grating and large condenser lenses. The stray infrared light present in this beam was found to be 3.8 per cent. Experiments 13 to 15 and 20 to 24 inclusive (Table III) were made with this improved light source.

The total energy flux of the beam in the position of the vessel was measured with a photonic cell fitted with a metal tube having a ground glass window (protected from the water with a plain glass window), and an internal reflecting cone. Its



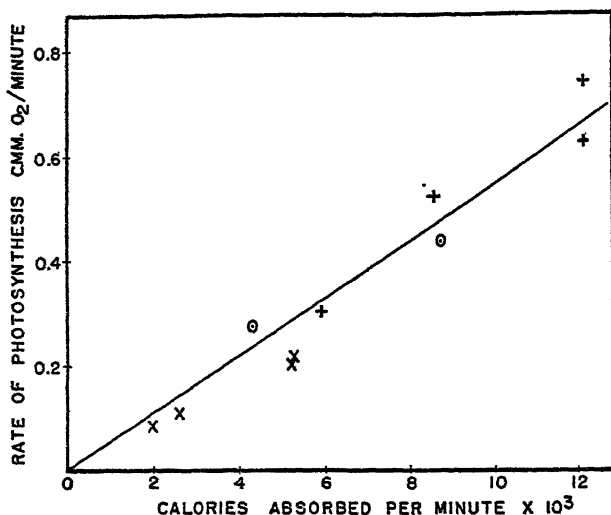


FIG. 4. The rate of *Chlorella* photosynthesis is plotted against the energy absorbed. Symbols  $\oplus$ , +, and  $\times$  represent Experiments 27, 28, and 29 respectively in Table III. Calories absorbed = intensity  $\times 5.09 \times F$ .

TABLE I

*The Wavelength Distribution of Light from a Tungsten Bulb Passing through 3 Meters of Water and a Jena RG5 Filter*

These values were calculated from spectrophotometric measurements of the filter transmission and from the data of Beber based on the International Critical Tables values of tungsten emission and water transmission.

Wavelength	Fraction of maximum
$m\mu$	per cent
650	0
660	15
670	41
680	92
685	100
690	95
700	65
710	27
720	11
750	2.3
800	7.7
900	0

6.8 per cent  
of the total  
energy

function was to increase the sensitive area of the photronic cell and to enable the cell itself to be kept out in the air while the intensity was measured below the water level. Experiments showed a decrease to about 90 per cent sensitivity near the edge

of the glass, hence only the central portion of the glass surface was used. The photronic cell was connected to a low resistance Type R Leeds and Northrup galvanometer with an Ayrton shunt made of resistance boxes. Each day the photronic cell was calibrated in its integrating box by exposure to the light beam at the same position at which the thermopile was used to measure the energy of the beam. A diaphragm with an opening  $1 \times 3$  cm. was placed in front of the photronic cell in the slightly converging light beam, and the galvanometer deflection noted. The photronic cell was removed and an Eppley "surface linear" thermopile was mounted so that the junctions were in the same plane in which the diaphragm had been in the photronic cell measurement, and by uniform lateral movement in from 5 to 10 positions, the average intensity of the beam was obtained. The thermopile in turn was calibrated daily against U. S. Bureau of Standards lamp C345 (checked also with lamp C149) which afforded an energy measurement in absolute units for the beam of light both outside the thermostat and incident upon the manometric vessel. The current source for the standard lamps was a 6 amp. 115 volt Westinghouse full wave rectifier with a filter circuit which reduced the ripple to about 0.5 per cent under operating conditions. The rectifier was fed from a voltage-regulating transformer. The voltage drop across the lamp was measured with a Weston d. c. voltmeter reputedly accurate to 0.5 per cent full scale. All light measurements and calibrations were carried out as soon as each experiment was completed. Calculated corrections were applied for reflection losses from glass and water surfaces and from the thermopile window whose transmission for the standard lamp radiation was taken as 0.85.

4. *The Measurement of the Fraction of Incident Light Absorbed by the Suspension.*—The authors are indebted to Dr. Foster Rieke for instruction in the use of the Ulbricht sphere. Our apparatus differed from that used by Dr. Rieke and will therefore be briefly described here. The integrating sphere consisted of two sheet metal hemispheres 18 inches in diameter. These hemispheres were coated on the inside with a white reflecting surface. The sphere was mounted above the optical bench on which a movable mirror was placed to throw most of the light beam through a lens mounted over a hole in the bottom of the sphere. The light beam came to a focus on the bottom of the manometric vessel which was suspended in the center of the sphere on movable horizontal wire arms (Fig. 3). A photoelectric tube and amplifier system were used to measure the light in the sphere. The phototube was placed in the side of the lower hemisphere and shielded from the light beam by a white baffle plate. In this way only the diffused light was measured. By moving the manometric vessel in and out of the beam, ( $D_0 = 100$ ), measurements of light intensities were made when the vessel was empty, when the vessel contained water ( $D_w$ ), when the vessel contained the Hill reagents ( $D_h$ ), when the vessel contained the Hill reagents plus the suspension ( $D_s$ ), and when the vessel contained India ink ( $D_i$ ). The fraction ( $F$ ) of the incident light absorbed was calculated from the formula:

$$F = 1 - \frac{D_s - D_i}{D_h - D_i}$$

The Ulbricht sphere should give essentially correct values for absorption by pigment alone, corrected for light scattering by particles, if used with monochromatic light. Since the light beam used in this work was not monochromatic, it was neces-

sary to make some estimate of the amount of error introduced into the absorption measurements as made with a selective receiver on a pigment having such sharp absorption bands as chlorophyll. The reason for this error is that the wavelength distribution of the beam is altered in passing through the chloroplast suspension. This gives rise to a higher proportion of light of longer wavelength in the beam after it has passed through the chloroplasts. The photocell used with the sphere was more sensitive to the wavelength range 690 to 720  $m\mu$  than to the range 660 to 690  $m\mu$ , thus the apparent transmission of the suspension was too great when measured by this procedure. Since the evaluation of the correction to be applied for this effect is not easy to deduce directly, it was approximated in a rough way and was found to be relatively small. An approximation of the error was obtained by the simultaneous measurement of a solution of purified chlorophyll in toluene with the photocell and with the thermopile. Since the wavelength maximum of chlorophyll absorption is shifted to shorter wavelengths in toluene from its position in the cells, this is only

TABLE II

*A Comparison of Thermopile and Photocell Measurements of the Absorption of a Chlorophyll Solution for the Red Light Used in the Experiments*

	Chlorophyll* in toluene	Wire screen†
Fraction absorbed, thermopile.....	0.316	0.536
Fraction absorbed, photocell.....	0.281	0.548
Error in photocell measurement, <i>per cent</i> .....	-7.9	+2.2

\* American Chlorophyll Company "5 x" in a 1 cm. layer of toluene.

† The measurements with the wire screen were made to test the agreement between photocell and thermopile readings with a non-selective absorber.

a rough method of estimating the error of sphere measurements with *in situ* chlorophyll. The light beam was filtered through 3 meters of water and the RG5 filter, passed through 1 cm. of the chlorophyll solution (or through a similar glass cell filled with toluene for the  $I_0$  reading), and then fell on the thermopile. The light not intercepted by the thermopile elements passed through the instrument and out the rear window of its housing where it was measured by the same photocell as was used in the sphere. Thus simultaneous measurements of the same beam were made with both the photocell and the non-selective thermopile. Transmission values of the chlorophyll solution as measured by both instruments are given in Table II. Assuming the thermopile measurements are correct we see that the use of the photocell gives values about 8 per cent too low for the fraction of light absorbed by the chlorophyll solution. Since this error is small as compared with the variability of plant material, and since it may not be quite correctly determined, no adjustment of the data was made to correct this error. Furthermore, Experiments 15a and 15b in Table III were both made with the same chloroplast suspension on the same day, but using different chloroplast concentrations (and hence different  $F$  values) and gave similar quantum yields. If there were any great error in the determination of  $F$ , the yield would certainly vary for different values of  $F$ .

TABLE III

*The Quantum Yield of the Oxygen Evolution of Chloroplast Suspensions in Vitro As Compared with the Photosynthesis of Chlorella pyrenoidosa Cells*

Experiment No.	Chloroplast chlorophyll	Light Intensity	Fraction of light absorbed	Quantum yield	Quanta required per O <sub>2</sub> molecule	Estimated error in measurement*
	mg./vessel	cal./cm. <sup>2</sup> /min. $\times 10^4$	F	$\gamma$	1/ $\gamma$	per cent
<i>A. Oxygen evolution by chloroplast suspensions†</i>						
Spinach:						
1	0.15	9.0	0.72	0.068	15	$\pm 10$
2a	0.15	6.6	0.71	0.068	16	5
2b	0.15	3.15	0.71	0.064	16	5
3	0.34	3.25	0.83	0.030	33	20
4	0.39	3.3	0.88	0.022	46	20
5	0.15	4.55	0.68	0.041	25	10
6	0.23	3.9	0.73	0.065	16	5
7	0.10	3.32	0.57	0.080	12	5
8	0.10	3.04	0.51	0.037	27	20
9	0.063	4.13	0.56	0.033	31	20
10	0.112	4.00	0.57	0.033	30	20
11a	0.103	4.18	0.545	0.013	36	20
11b	0.103	3.85	0.545	0.026	39	20
11c	0.103	3.58	0.545	0.028	78	50
12a	0.17	4.18	0.70	0.030	33	5
12b	0.17	3.85	0.70	0.030	33	5
12c	0.17	3.58	0.70	0.030	34	5
13	0.09	1.6	0.33	0.058	17	20
14	0.15	2.8	0.83	0.053	19	5
15a	0.045	2.8	0.47	0.031	31.8	5
15b	0.090	2.8	0.79	0.036	28.0	5

Average.. 0.042

S. E.  $\frac{1}{2}$  =  $\pm 0.0040$  or  $\pm 9.5$  per cent*Tradescantia:*

16	0.195	4.6	0.77	0.03	33	50
17	0.157	7.7	0.56	0.024	41	20
18	0.23	2.25	0.74	0.065	15	5
19	0.075	1.85	0.70	0.017	59	10

\* The error was estimated more or less arbitrarily to enable us to record those experiments in which the manometric or/and intensity measurements were less satisfactory because of minor errors of operation.

† The gas space of the manometric vessel was filled with air for all experiments except Nos. 1 and 2, for which nitrogen was used. The side arms of the manometric vessels were filled with 0.5 ml. of a 10 per cent NaOH solution for all spinach and *Tradescantia* experiments. In an experiment in which the rate of uptake of CO<sub>2</sub> liberated from carbonate with acid was measured, this amount of NaOH in the side arm was found to absorb CO<sub>2</sub> 6 to 8 times as fast as the most rapid evolution of oxygen found in this work, hence CO<sub>2</sub> evolution, if any, was a negligible factor in the observed results.

TABLE III—*Concluded*

Experiment No.	Chloroplast chlorophyll	Light Intensity	Fraction of light absorbed	Quantum yield	Quanta required per O <sub>2</sub> molecule	Estimated error in measurement*
	mg./vessel	cal./cm. <sup>2</sup> /min. × 10 <sup>3</sup>	F	γ	1/γ	per cent
<i>Tradescantia:</i>						
20	0.10	2.12	0.80	0.03	34	10
21	0.075	2.90	0.81	0.024	42	10
22	0.075	3.30	0.80	0.025	39	10
23	0.075	2.18	0.72	0.019	53	20
24	0.060	2.30	0.47	0.032	31	10

Average.. 0.030

S. E. § = ±0.005 or ± 17 per cent

*B. Photosynthesis by Chlorella pyrenoidosa*||

25	—	13.5	0.35	0.096	10	20
26	—	7.51	0.31	0.063	16	20
27a	—	4.37	0.39	0.098	10	10
27b	—	2.24	0.39	0.116	8.6	10
28a	—	3.72	0.28	0.069	15	10
28b	—	1.86	0.28	0.080	13	10
28c	—	1.34	0.28	0.080	13	10
28d	—	3.72	0.28	0.077	13	10
29a	—	4.18	0.57	0.096	11	10
29b	—	2.04	0.57	0.097	10	10
29c	—	2.93	0.57	0.116	8.6	10
29d	—	4.18	0.57	0.113	8.8	10

Average.. 0.092

S. E. § = ±0.005 or ± 5.4 per cent

The sodium sorbitol borate buffer was added as a 2.0 M stock solution of pH 6.5 in Experiments 1 to 5 and 16 to 18 and of pH 6.8 in Experiments 6 to 15 and 19 to 24. It was found that upon dilution of this buffer with the Hill solution as used in the manometric vessels, the pH shifted to 7.4 and 7.7 respectively. Inspection of Table III shows no significant difference in yield between these two pH values. Whether or not this slightly alkaline buffering was disadvantageous at the low light intensity used in this work was decided as follows: Two quantum yield measurements were made on the same suspension of spinach chloroplasts under identical conditions, one at a pH of 7.7 and the other at pH 7.0. The yields for these were respectively 0.020 and 0.022 molecules of oxygen per quantum of light energy. Repetition of this experiment on another day gave values of 0.027 and 0.023 molecules of oxygen per quantum respectively for pH 7.7 and 7.0. It would appear that at the low light intensity used in this study, buffering at pH 7.7 gives quantum yield values which are not significantly different from values obtained when buffering at pH 7.0. Holt and French (1945) found that at high light intensity similar chloroplast suspensions gave a maximum oxygen evolution at pH 7.0.

§ The standard error for a small number of samples:

$$S.E. = \sqrt{\frac{\sum (\bar{X} - X)^2}{N(N-1)}}$$

|| Experiments 25 and 26 were made with single Warburg manometers while all other experiments were made with the differential manometer. The buffer used for the *Chlorella* experiments was Warburg's carbonate No. 9 (15 ml. 0.1 M Na<sub>2</sub>CO<sub>3</sub> plus 85 ml. 0.1 M KHCO<sub>3</sub>). The algae were suspended directly in this mixture.

## RESULTS

The quantum yield data are summarized in Table III. There is considerable variation in the quantum yields, particularly in those of the spinach, and to a lesser extent in the yields of the *Tradescantia* chloroplast suspensions. The quantum yield for oxygen production by spinach varied from 0.013 to 0.080 molecule per quantum with an average yield of 0.042 and a standard error of  $\pm 0.004$ . Yields for *Tradescantia* varied from 0.017 to 0.065 with an average yield of 0.030 and a standard error of  $\pm 0.005$ . For *Chlorella* photosynthesis, quantum yields varied from 0.063 to 0.116 with an average of 0.092 and a standard error of  $\pm 0.005$ . Although the quantum yields of the spinach and *Tradescantia* chloroplasts are less than the yields for *Chlorella* photosynthesis, the results could possibly still be taken to suggest that the photochemical reaction studied was more closely related to photosynthesis than to an oxidation reaction, since occasional spinach and *Tradescantia* chloroplast experiments gave results comparable to the values for algal photosynthesis.

No accurate measurements were made to determine how much of the variation in quantum yields was operational error and how much was the result of the previous treatment of the test material, especially in case of the spinach which was obtained at a grocery store. One line of evidence however which lends credence to the belief that operational error was small is as follows: When part of a particular lot of spinach was stored in the refrigerator, a suspension made from this material on the following day, and its quantum yield compared with that of the previous day, good agreement was found (Experiments 9, 10 and 13, 14 in Table III). However, upon standing longer, quantum yields were definitely lower. This is shown in Experiments 13, 14, and 15 in Table III, which represent 1st, 2nd, and 3rd day runs on the same test material, the spinach being kept in the cold room, in the dark, and well moistened between runs. The yields for the respective days were: 0.058, 0.053, and 0.036 molecule per quantum. It is obvious that because of this decrease in activity and because of the difficulty of knowing the exact state of the market spinach, that this material is not as desirable as material such as *Tradescantia* which was obtained fresh from the greenhouse. Market spinach is worth further investigation however, when one considers the difficulty in obtaining other material in sufficient quantity all year.

## DISCUSSION

It seems evident from the good agreement of the several duplicate determinations with the same suspension that the variability of the results is due to variation of the state of the plant material rather than to any great inaccuracy in the technique of measuring either light intensities or pressure changes. There is no correlation of yield with the fraction of light absorbed. A

correlation might be expected in event of a systematic error in absorption measurements. The values obtained for *Chlorella* photosynthesis agree reasonably well with values obtained in the far more elaborate studies of Emerson and Lewis (1943), Manning, Stauffer, Duggar, and Daniels (1939), and Rieke (1939, 1941), thus providing a rough check on our entire procedure. The present results for the Hill reaction give an average value of about 0.04 molecule of oxygen per quantum of light absorbed. Several individual values in the neighborhood of 0.07 were found. In general it may be said that the efficiency of the *in vitro* oxygen production by chloroplasts is less than that of normal *Chlorella* photosynthesis (0.09) but of the same general order of magnitude. It appears that about two or three times as much light is required by isolated chloroplasts in Hill's solution under our conditions for a given amount of oxygen production as is needed for photosynthesis by intact algal cells. What then becomes of this extra light? Mechanisms proposed to account for the utilization of four to ten quanta of light for the liberation of an oxygen molecule by photosynthesis become of doubtful value when stretched to accommodate twenty or thirty quanta. It would appear more likely that the larger part of the light in this case is absorbed by molecules of chlorophyll that do not cause chemical reaction but merely transform the light into heat. The inability of certain molecules of chlorophyll to initiate the Hill reaction could easily be accounted for by a lack of a sufficient concentration of the enzymes or other essential substances in the immediate neighborhood of the activated chlorophyll. It may be, however, that there are some chloroplasts which are entirely inactive, as well as some that function with their full theoretical efficiency. It is to be noted that some of the measurements with isolated chloroplasts give values approximating the yields found for normal photosynthesis. This might perhaps be taken as a suggestion that the Hill reaction, when it takes place in chloroplasts having largely active chlorophyll gives off oxygen with the same efficiency as in the common photosynthetic reaction. The present results, in spite of their variability, definitely show that the Hill reaction is less efficient than the photooxidation of organic compounds by chlorophyll in organic solvents, probably more efficient than photooxidation in leaves, and about one-half as efficient as photosynthesis in algal cells. Table IV summarizes the quantum yield data for photoactivated chlorophyll in various reactions.

In order to make more accurate measurements of the true quantum yield uncomplicated by the variation in the state of the leaves, the physiology of the Hill reaction must be investigated further. In particular, more information as to the effect of pretreatment of the leaves on the rate of the oxygen evolution at both high and low intensities must be obtained before better quantum yield experiments can be made. We suspect on the basis of the work of Kumm and French (1945) that refinements in the procedure of leaf treatment before

chloroplast isolation might lead to an efficiency of the Hill reaction more nearly comparable to that of photosynthesis.

The obvious experimental approaches to further studies of the energetics of the Hill reaction are to follow the effect on the efficiency of: (a) Pretreatment of the leaves from which chloroplasts are extracted, (b) variation of the extraction procedure and the concentration of the reactants in the Hill solution, (c) fractionation of ground chloroplasts in an attempt to concentrate the active constituents. Much useful information should also be obtained by correlating the maximum rate of oxygen formation in bright light with the yield per quantum of light of low intensity.

TABLE IV  
*Quantum Yields of Various Photochemical Reactions Involving Chlorophyll*

Reaction	State of chlorophyll	Quantum yield	Worker
Photooxidation of allyl thiourea....	Solution in acetone	1.0	Gaffron
Photooxidation of allyl thiourea....	Solution in acetone	0.4	Brown (unpublished, this laboratory)
Photooxidative bleaching of chlorophyll .....	Solution in acetone or benzene	$5 \times 10^{-4}$	Aronoff and Mackinney
Photooxidation of leaf constituents..	In leaf cuttings	0.01	Franck and French
Photosynthesis.....	In algal cells	0.09	Various (see text)
Hill reaction.....	In chloroplasts	0.04	Present paper

#### SUMMARY

1. The quantum yield of oxygen liberation by spinach and *Tradescantia* chloroplasts suspended in solutions containing ferric oxalate and potassium ferricyanide varied from 0.013 to 0.080.

2. It was concluded that the nature of this oxygen liberation reaction is not fundamentally different from the formation of oxygen in normal photosynthesis, with respect to its light efficiency.

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# DIFFERING RATES OF DEATH AT INNER AND OUTER SURFACES OF THE PROTOPLASM

## III. EFFECTS OF MERCURIC CHLORIDE ON *NITELLA*

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(Received for publication, September 29, 1944)

It is a striking fact that when formaldehyde is applied to *Nitella* death occurs sooner at the inner protoplasmic surface than at the outer if the external solution contains relatively little potassium. It would seem possible to produce this effect by means of certain other substances which quickly penetrate to the inner surface. The present paper shows that mercuric chloride is such a substance.

The cells used were 3 or 4 inches long and the reagent covered a region (*A*) 1 cm. long near one end of the cell. The potential between this and a similar area (*B*) at the other end of the cell, in contact with 0.01 *M* KCl, was recorded.<sup>1</sup>

Fig. 1 shows the result of applying 0.01 *M* HgCl<sub>2</sub> at *A*. At the start the spot was in contact with 0.001 *M* NaCl and there was a positive<sup>2</sup> P.D. of 100 mv. due chiefly to the diffusion potential of KCl at the inner non-aqueous protoplasmic surface, *Y*. The loss of this potential as shown by the rise of the curve indicates that the reagent penetrated through the non-aqueous outer protoplasmic surface *X* and the aqueous layer of the protoplasm *W*, and affected the inner protoplasmic surface, *Y*.<sup>3</sup>

Under the influence of the reagent the inner surface *Y* became completely permeable to KCl and other electrolytes so that the curve rose to zero.

With 0.01 *M* HgCl<sub>2</sub> this occurs in less than 1 minute: with 0.001 *M* HgCl<sub>2</sub> the rise is usually slower. These changes are irreversible.

<sup>1</sup> The cells, after being freed from neighboring cells, stood in the laboratory at 15°C. ± 1°C. in Solution A (cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, **17**, 87) for several days. Fig. 1 refers to cells in Lot B, Fig. 2 to cells in Lot A (cf. Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312).

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, **21**, 541). Temperature 20-26°C. Regarding the amplifier see the reference just cited.

The solutions here used did not produce plasmolysis.

<sup>2</sup> The P.D. is said to be positive when the positive current tends to flow from the sap across the protoplasm to the external solution.

<sup>3</sup> Regarding potentials at *X* and *Y* see Osterhout, W. J. V., *J. Gen. Physiol.*, 1944-45, **28**, 23.

After  $I'$  has become completely permeable to  $KCl$ , as shown by the rise of the curve to zero, we find that the outer non-aqueous surface  $X$  has not yet become completely permeable since it shows a change in P.D. when we vary the concentrations of electrolytes in contact with it (concentration effect).

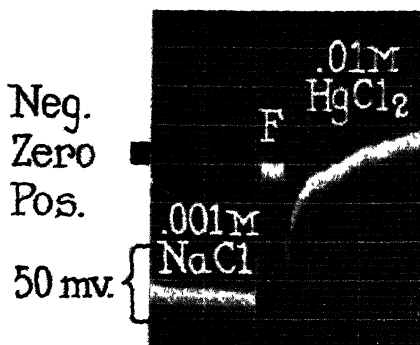


FIG. 1. When  $HgCl_2$  was applied there was a loss of potential, as shown by the rise of the curve.

The record shows the P.D. at a spot  $A$  which was connected to a spot  $B$  whose P.D. was zero owing to the application of  $0.01\text{ M } KCl$ .

At the start the spot  $A$  was in contact with  $0.001\text{ M } NaCl$  and had a positive P.D. of  $100\text{ mv.}$  When the solution was removed from  $A$ , thus breaking the electrical circuit, the curve jumped to  $F$ , the "free grid" value of the amplifier.

When  $0.01\text{ M } HgCl_2$  was applied and the spot  $A$  was again in the circuit the curve rose approximately to zero indicating a complete loss of potential at the inner protoplasmic surface,  $I'$ . But the outer protoplasmic surface  $X$  had not become completely permeable for subsequent portions of the record (not reproduced here) showed marked alterations in P.D. at the outer surface when the solutions were changed (these do not occur in a dead cell).

The cell was freed from neighboring cells and kept in Solution A for 4 months at  $15^\circ C. \pm 1^\circ C.$  An hour before the experiment was started the temperature was raised to  $25^\circ C.$

Time marks 15 seconds apart.

Before applying  $HgCl_2$  the average change of P.D. on replacing  $0.01\text{ M } KCl$  by  $0.001\text{ M } KCl$  was  $41 \pm 2.3\text{ mv.}$  (7 observations). This value became  $27 \pm 1.4\text{ mv.}$  (6 observations) after the curve had risen to zero under the influence of  $0.01\text{ M}$  or of  $0.001\text{ M } HgCl_2$ . The corresponding values for  $NaCl$  are  $46 \pm 1.3\text{ mv.}$  (7 observations) before and  $29 \pm 5.8\text{ mv.}$  (6 observations) after application of the reagent. These effects gradually disappear.

Hence if we employ permeability as a test of death we may say that death arrives sooner at the inner protoplasmic surface  $I'$  than at the outer,  $X$ .

Since this may be due, in part at least, to unlike conditions at  $X$  and  $I'$  an attempt was made to make these conditions more nearly equal by raising the

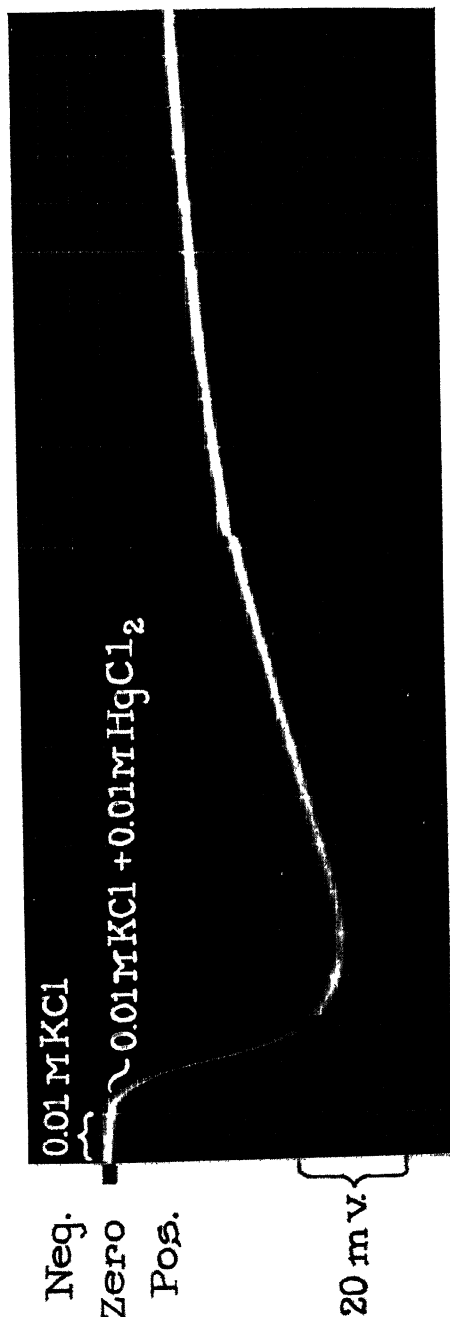


FIG. 2. Shows that when the outer protoplasmic surface  $X$  was in contact with  $0.01\text{ M KCl}$  it was affected sooner by the addition of  $\text{HgCl}_2$  than was the inner protoplasmic surface  $Y$ .

At the start the recorded potential at  $X$  which was equal to the positive potential at  $Y$  so that the total potential was zero (the spot  $A$  was connected through the galvanometer to another spot  $B$  whose p.d. was kept at zero by the application of  $0.01\text{ M KCl}$ ).

When  $\text{HgCl}_2$  was applied,  $X$  was affected first and the loss of negative potential at  $X$  caused the curve to fall. Later the positive potential at  $Y$  began to fall off more rapidly than the negative potential at  $X$  and the curve began to rise. Eventually the curve reached zero (not shown in the figure) indicating a total loss of potential. In this case the change of solutions was made without breaking the electrical circuit since a continuous flow was maintained (cf. Hill, S. E., and Osterhout, W. J. *V., J. Gen. Physiol.*, 1937-38, 21, 541).

The cell was freed from neighboring cells and kept for 24 hours in tap water at  $15^\circ\text{C.} \pm 1^\circ\text{C}$ . An hour before the experiment was started the temperature was raised to  $25^\circ\text{C}$ .

Time marks 5 seconds apart.

The time of death was longer than in Fig. 1. This may be due in part to the fact that the cells were from different lots collected in different localities.

concentration of KCl in contact with  $X$  to 0.01 M since the concentration of KCl in the sap in contact with  $I'$  is about 0.05 M. It was then found that death occurred sooner at  $X$  than at  $I'$ , as shown in Fig. 2. Here the applied reagent was 0.01 M  $\text{HgCl}_2$  + 0.01 M KCl which produced at the start a p.d. of zero mv. due to an outwardly directed (positive<sup>a</sup>) p.d. at  $I'$  balanced by an equal negative p.d. at  $X$  (both due to the diffusion potential of KCl).

In this case  $X$  was affected first,<sup>4</sup> as shown by the loss of negative potential and the consequent fall of the curve: later the action on  $I'$  began to predominate so that the curve began to rise. It reached zero when both  $X$  and  $I'$  had become completely permeable. This change was irreversible.

During the gradual loss of potential at  $I'$ , as shown in Fig. 1, there may be no great change at  $X$  in respect to permeability, as indicated by the concentration effect. But at the same time the potassium effect falls off showing that  $X$  has been altered. The potassium effect is the change in p.d. in a positive direction when 0.01 M KCl is replaced by 0.01 M NaCl. Before treatment with  $\text{HgCl}_2$  it was  $31 \pm 2.8$  mv. (7 observations). After  $\text{HgCl}_2$  had caused the curve to rise to zero the potassium effect disappeared in most cases and in others had a very low value. This change is presumably due to loss of a group of organic substances, called for convenience  $R$ , as described in previous papers.<sup>5</sup>

The results present a striking parallel to those obtained with formaldehyde as described in a former paper.<sup>6</sup> It is evident that in both cases the rate of death may differ at the inner and outer surfaces of the protoplasm and that this can be controlled by changing the experimental conditions.

It may be added that earlier experiments<sup>7</sup> with chloroform gave a similar result in showing that the concentration of KCl determines the behavior. The solutions were saturated with chloroform in all cases. When the external solution contained 0.05 M KCl or less  $I'$  was killed first but when it contained 0.1 M KCl  $X$  was killed first.

Even when  $X$  and  $I'$  are under the same conditions they do not behave alike in all respects. When both are in contact with sap the difference in p.d. between the two surfaces is about<sup>8</sup> 15 mv.

<sup>4</sup> This is presumably because the reagent reaches  $X$  first. As might be expected, the distance to which the curve descends varies greatly with different cells.

<sup>5</sup> Cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, **17**, 87, 99, 105.

<sup>6</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1944-45, **28**, 23.

<sup>7</sup> Osterhout, W. J. V., Some aspects of permeability and bioelectrical phenomena, in Molecular physics in relation to biology, *Bull. Nat. Research Council*, No. 69, 1929, 170.

<sup>8</sup> Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, **11**, 391.

## SUMMARY

The inner and outer protoplasmic surfaces in *Nitella* may differ greatly in behavior. When 0.01 M  $\text{HgCl}_2$  is applied externally death arrives first at the inner surface. But when 0.01 M  $\text{HgCl}_2$  + 0.01 M KCl is applied death takes place sooner at the outer surface. Since 0.01 M KCl by itself is not toxic its effect may be to condition the surface layer chemically or by means of the diffusion potential it sets up (this may amount to 100 mv.).

These surfaces consist of non-aqueous films forming the boundaries of a layer of aqueous protoplasm not over 10 microns in thickness.

These and earlier experiments with formaldehyde and with chloroform show clearly that it is possible to control the behavior of the protoplasmic surfaces so that when a toxic agent is applied it may produce death more rapidly at the inner or at the outer surface according to experimental conditions.



# THE MECHANISM OF THE INHIBITION OF HEMOLYSIS

## II. EFFECTS OF pH ON INHIBITION BY PLASMA AND BY CHOLESTEROL SOLS

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The way in which the inhibitory effects of plasma and of cholesterol sols depends on the pH of the hemolytic systems to which they are added is interesting for two reasons. (1) The inhibitory effect of cholesterol sols and part of the inhibitory effect of plasma seem to be due to the inhibitor competing with the red cells for the lysin in the system. It has sometimes been assumed, although without direct evidence, that the reaction between the lysin and the inhibitor involved in this competition is similar to that which takes place between the lysin and a component of the red cell surface; *e.g.*, the fact that cholesterol is able to compete for lysin in a system containing digitonin suggests that a reaction between digitonin and the cholesterol of the red cell surface is involved in the hemolytic process. There have been several investigations on the effect of pH on the lytic process, but none on its effects on the inhibitory processes. (2) Changing the pH of the hemolytic system provides a means of comparing, and also, as it turns out, of contrasting, the inhibitory effects of cholesterol sols, which are almost exclusively the result of reactions which take place in the bulk phase of the system, with those of plasma, which are the result of reactions taking place not only in the bulk phase, but also in the neighborhood of the cell surfaces.

### Methods

A series of stock buffers were prepared by adding 250 ml. of 0.067 M  $\text{KH}_2\text{PO}_4$  and 0.067 M  $\text{Na}_2\text{HPO}_4$ , mixed in various proportions to give pH's of 5.6, 6.4, 6.8, 7.2, and 8.6, to 750 ml. of 1.2 per cent NaCl. By further mixing these stock buffers with each other in various proportions, an isotonic solution of any pH within the range can be obtained, and the pH's were verified with the glass electrode.

*Red Cell Suspensions.*—These were of "standard" concentration; *i.e.*, the red cells of 1 ml. of human blood, received into citrate, washed three times with saline (1 per cent NaCl), and finally suspended in 20 ml. of saline.

*Inhibitors.*—Human plasma, diluted 1 in 100, 1 in 200, etc., with saline was used throughout. The cholesterol sols were made by the method described by Lee and Tsai (1942), and were diluted with saline (1 in 10 · · · 1 in 100) just before use.

*Standard Systems.*—At any one pH, the standard system was formed by 0.8 ml. of various dilutions of lysin dissolved in buffer at that pH, 0.8 ml. of saline, and 0.4 ml. of cell suspension. The times for complete lysis were measured at 37°C. In the case of sodium taurocholate, the procedure was altered somewhat because the dissolving of the lysin in the buffer solutions produced changes in the pH of some of them; 0.1 N



TABLE I

*Inhibition (R-Values) of 1 in 20,000 Saponin and 1 in 67,000 Digitonin by Plasma and by Cholesterol Sol: 37°C., Time of Contact, 24 Hours*

System	Dilution of inhibitor	pH				
		5.6	6.4	6.8	7.2	8.6
Saponin.....	—	1.0	1.3	0.8	3.1	4.0
Saponin plus plasma.....	1/200	2.3	2.6	1.7	2.2	2.5
Saponin plus cholesterol sol.	1/40	1.9	2.0	1.5	2.1	2.4
Digitonin.....	—	1.0	0.9	1.0	1.0	1.1
Digitonin plus plasma.....	1/800	1.2	1.4	1.5	1.6	1.2
Digitonin plus cholesterol sol.....	1/320	1.8	1.6	1.8	1.8	1.9

TABLE II

*Inhibition (R-Values) of 1 in 2,000 Sodium Taurocholate by Plasma and by Cholesterol Sol: 37°C., Time of Contact, 24 Hours*

System	Dilution of inhibitor	pH						
		5.3	5.6	6.0	6.5	7.0	7.5	8.5
Taurocholate.....	—	—	1.0	1.1	—	1.9	2.6	3.5
Taurocholate plus plasma....	1/500	1.2	—	1.2	—	1.2	—	1.3
Taurocholate plus cholesterol sol.....	1/10	—	1.1	1.2	1.3	—	1.5	1.7

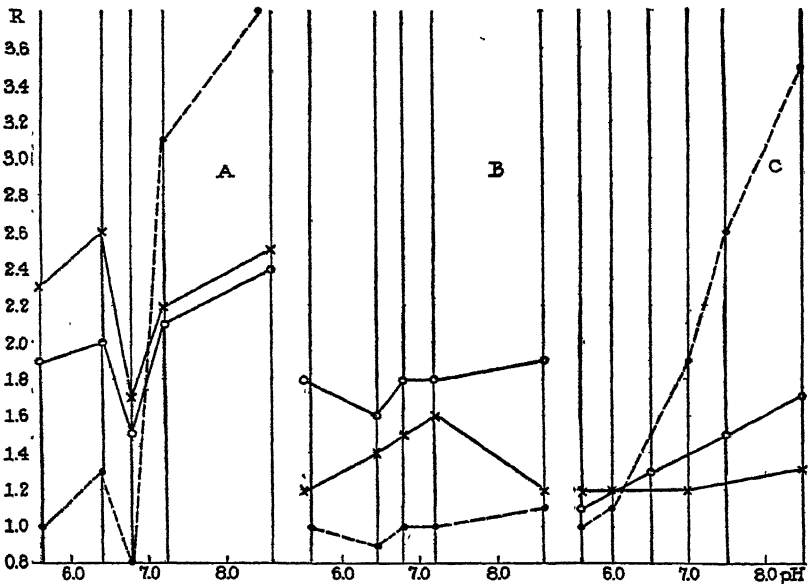


FIG. 1. R-values plotted against values of pH for systems containing saponin (A), digitonin (B), and sodium taurocholate (C). Dotted lines, lysin acting alone; crosses, with plasma present as an inhibitor; circles, with cholesterol sol present as an inhibitor. For explanation, see text.

NaOH was accordingly added to bring the pH to convenient values, measured electrometrically.

*Systems Containing Inhibitors.*—At any one pH, these consisted of 0.8 ml. of various dilutions of lysin in buffer at that pH, and 0.8 ml. of diluted plasma or diluted cholesterol sol, as the case might be. The tubes containing the mixtures were allowed to stand at 37°C. for 24 hours ("time of contact") in order for any reactions between lysin and inhibitor to reach equilibrium, and 0.4 ml. of cell suspension was added at the end of the time. By comparing the times for complete lysis with those for the corresponding standard system, the inhibition produced by the plasma or the cholesterol sol can be obtained either as  $R$ -value or as a  $\Delta$ -value (Ponder, 1943). For convenience in comparison, Tables I and II and Fig. 1 show the results in terms of  $R$ -values, although there is evidence that most of the inhibitory effects of cholesterol sols and some of the inhibitory effects of plasma are due to reactions which take place between the inhibitors and the lysin in the bulk phase of the system ( $\Delta$ -reactions); the equation

$$\Delta = c_1 - c_1/R,$$

however, enables  $\Delta$  to be calculated from the  $R$ -values. It is important to notice that both  $R$  and  $\Delta$  are functions not only of the quantity of inhibitor used, but also of the concentration of lysin  $c_1$  initially present in the system (see Ponder, 1943), and so comparisons of the inhibitory effects should always be made for the same lysin concentration.

## RESULTS

These are shown in Tables I and II, which are self-explanatory, and are also represented graphically in Fig. 1. They can be discussed from several points of view.

1. The interpretation of the results obtained for systems containing inhibitors such as plasma and cholesterol sols, which react, in part at least, with the lysin in the bulk phase of the system, depends on the extent to which we are able to analyze the simultaneous reactions between cells and lysin on the one hand and between inhibitor and lysin on the other. A little progress has already been made in this direction. When cells are added to lysin in concentration  $c_1$ , a quantity of lysin equal to  $\zeta c_1$  becomes concentrated at the cell surfaces, leaving free a quantity  $c_2$ , equal to  $c_1(1 - \zeta)$ . Somewhat limited observations have shown that  $\zeta$  is a constant, the value of which depends on the nature of the lysin, and probably also on the type of red cell. As hemolysis proceeds, the quantity of free lysin  $c_2$  decreases in such a way that the amount left free at a time corresponding to complete lysis is a constant fraction of  $c_1$ , the decrease also apparently being substantially linear with time (Ponder, 1934, 1945).

If an inhibitor such as plasma or cholesterol sol is present in the system, a reaction also occurs between it and the free lysin, so that a quantity of lysin  $\Delta$  is rendered inert. In spite of much investigation (Ponder, 1924, 1925, 1943, Ponder and Gordon, 1934), no satisfactory expression for the relation between

$\Delta$  and  $c_1$  or  $c_2$  has been found, but as a first approximation let us assume that  $\Delta$  is equal to  $\alpha c_2$ . The values of  $\Delta$  obtained experimentally are found to depend on the *concentration of the red cells added to the hemolytic system*,<sup>1</sup> and this leads to the further conclusion that the reaction between lysin and inhibitor is partially reversible. In any system containing lysin, cells, and inhibitor, we accordingly have a distribution of the lysin into three quantities: the amount of lysin concentrated in the region of the cell surfaces and entering into reactions there, the amount left free, and the amount inhibited as a result of a reaction with the inhibitor. When red cells are added to a system containing lysin and inhibitor, the relations of these three quantities will be, approximately,

Concentrated at surfaces .....	$\xi c_1/1 + \xi + \alpha$
Free .....	$c_1/1 + \xi + \alpha$
Inhibited .....	$\alpha c_1/1 + \xi + \alpha$

Consider the effect of a change in the pH of a lysin-cell system which results in a change in hemolytic activity corresponding to a change in  $\xi$ ; *i.e.*, to a change in the "affinity" of the red cell surface for the lysin. If an inhibitor is present at the same time, a competition of the cell surfaces and the inhibitor for the lysin will occur, and in the simplest cases the amount of inhibition observed will tend to be small at the pH's at which the lytic activity is great, and *vice versa*. Suppose, for example, that the value of  $\xi$  in a cell-lysin system containing lysin in initial concentration  $c_1$  is 0.3 at pH 5.6 but 0.1 at pH 8.6; the activity of the lysin will then be greater at the former pH than at the latter, and the value of  $R$  for the system at pH 8.6 will be approximately 3 times that for the system at pH 5.6. If we add an inhibitor which has the same affinity for the lysin at the two pH's, *e.g.*,  $\alpha = 0.25$ , the change in  $\xi$  which occurs as the pH changes from 5.6 to 8.6 will result in an increase in  $\Delta$ , the amount of lysin apparently inhibited, from  $0.161 c_1$  to  $0.186 c_1$ , or 1.15 times, although the reaction by means of which this inhibition is produced has no pH dependence of its own. In this way there will arise a tendency for an increase in lytic activity with change in pH to be associated with decreased inhibition by an added inhibitor, whereas decreases in lytic activity will be associated with increases in inhibition. This is noticeable in the cases of the three curves of Fig. 1A (lysin activity,<sup>2</sup> plasma inhibition, and cholesterol sol inhibition), two out of the three of Fig. 1B (lysin activity and cholesterol sol inhibition), and two out of the three of Fig. 1C (lysin activity and cholesterol sol inhibition). In the absence of any means of analyzing the situ-

<sup>1</sup> This point has been discussed by Ponder and Gordon (1934), for systems in which the inhibitor is plasma. Very similar results, indicating some degree of reversibility of the lysin-inhibitor reaction, can be obtained when the inhibitor is cholesterol sol, and when the concentration of red cells in the system is varied.

<sup>2</sup> The way in which the lytic activity of saponin varies with pH is substantially that described by Bodansky (1929).

ation more completely, we may suspect that in these cases the variations in the inhibition observed at different pH's are only reflections of variations in the activity of the lysins with pH.<sup>3</sup>

2. The changes in the inhibitory power of plasma for digitonin hemolysis (Fig. 1B, crosses), however, cannot be explained in this way. The activity of

<sup>3</sup> A test of the validity of this way of approaching the problem is provided by systems in which the inhibitor is a suspension of red cell ghosts (stromata). These are as similar to the red cells themselves as any material is likely to be. The suspension of ghosts is prepared by hemolyzing red cells with water, saturating with CO<sub>2</sub>, and washing the ghosts several times with 0.1 per cent NaCl saturated with CO<sub>2</sub> (Ponder, 1940). The ghosts are finally suspended in 1 per cent NaCl in a concentration such that 1 ml. of the suspension is equivalent to 1 ml. of standard red cell suspension in its ability to react with lysin (saponin). The method of determining this equivalence is described by Ponder (1934). Systems containing 0.8 ml. of 1 in 10,000 saponin at various pH's and 0.8 ml. of the ghost suspension diluted 1 in 10 are allowed to stand at 37°C. for 24 hours, at the end of which time 0.4 ml. of red cell suspension is added and the times for complete hemolysis determined. These times are converted to *R*-values by reference to time-dilution curves at 37°C. for cell-lysin systems at the various pH's. The variation in activity of the saponin at the different pH's is expressed (as in Tables I and II) by referring to the cell-lysin system at pH 5.6, for which *R* is put as 1.0.

TABLE III

System	pH				
	5.6	6.2	6.8	7.2	8.6
Saponin . . . . .	1.0	1.2	1.3	3.0	3.6
Saponin plus ghosts . . . . .	8.0	6.0	4.1	2.7	2.3

The results obtained with saponin are shown in Table III. It will be noticed that the pH dependence for 1 in 10,000 saponin is not quite the same as that for 1 in 20,000 saponin (Table I); this difference was also remarked upon by Bodansky (1929).

In a general sort of way, the inhibition produced by the ghosts is greatest at the pH's at which the lytic activity is greatest, and *vice versa*; moreover, the ratio of the lytic activities at pH's 5.6 and 8.6 is 3.6, and the ratio of the inhibitory effects of the ghosts at the same two pH's is 1/3.5. Too much emphasis should not be placed on this, for the *R*-values in the two rows of Table III are not linear with each other, but the result of the experiment shows that the pH dependence of the inhibition produced by the ghosts is of the same general form as that of the hemolytic effect; *i.e.*, that the ghosts compete for the lysin in the system in the same sort of way as the red cell surfaces do. This observation gives one a confidence in the method of analyzing the results which one might otherwise not have had. In addition, the experiment shows that the pH dependence of the inhibition produced by ghosts is quite different from that of the inhibition produced by either plasma or cholesterol, neither of which compete with the red cell surface for lysin in the same way as the ghosts do.

the lysin in a cell-lysin system is almost constant between pH 5.6 and pH 8.6, whereas the inhibition produced by plasma rises to a distinct maximum at pH 7.2.

The presence of this maximum throws considerable light on the hypothesis that the inhibitory power of plasma is largely due to its contained cholesterol (Ransom, 1901, Tsai and Lee, 1941, Yi and Meng, 1941, and their collaborators). The way in which the inhibition produced by plasma, as compared with that produced by cholesterol sol, varies with pH makes this conclusion difficult to accept without a great deal of qualification. The  $R$ -value for the inhibition by plasma diluted 1 in 800 is 1.2 at pH 5.6, while that produced by cholesterol sol diluted 1 in 320 is 1.8; calculating on the basis of  $R$  being linear with  $Q$ , an  $R$ -value of 1.8 at pH 5.6 would be obtained with plasma diluted 1 in 200.<sup>4</sup> By the same type of calculation, a 1 in 200 plasma would give an  $R$ -value of 3.4 at pH 7.2, while at this pH the inhibition produced by the cholesterol sol amounts to only  $R = 1.8$ . A hypothesis which says that the inhibitory power of plasma is almost entirely due to its contained cholesterol accordingly cannot be correct for all pH's, if indeed it is correct for any. The simplest explanation of the observations is that inhibitors other than cholesterol are present in plasma (Lee and Tsai, 1942, Ponder, 1944) and that their effects reach a maximum at pH 7.2.<sup>5</sup>

The results obtained when the lysin is sodium taurocholate lead to the same sort of conclusion, although in this case it is the inhibitory power of plasma which is almost constant over the pH range. A quantity of plasma, equal in inhibitory power to a specified quantity of cholesterol sol at pH 8.6, would be much more inhibitory than the cholesterol sol at pH 5.6. This is what would happen if plasma were to contain inhibitors other than cholesterol, with their maximum inhibitory power in the neighborhood of pH 5.6.

<sup>4</sup> It may be asked why this calculation, with its assumption, is necessary, and why experimental results are not presented instead. The reason is that if we were actually to use a plasma dilution of 1 in 200 ( $R = 1.8$ , to be compared with  $R = 1.8$  for the cholesterol sol diluted 1 in 320) at pH 5.6, it would be impossible to measure the  $R$ -value for the inhibition (greater) at pH 7.2, for instance. This difficulty, which has its origin in the non-linear form of the time-dilution curve, will be familiar to those who have worked with hemolytic systems.

<sup>5</sup> In these experiments, the cholesterol is in a micellar state (mean diameter of the micelles, about  $0.1\mu$ ), and the physical state in which it is present in plasma may be very different. The inhibitory power of the sol nevertheless seems to be about the same as that of the cholesterol in plasma, for the sol diluted 1 in 10 has about the same inhibitory effect on saponin as has plasma diluted 1 in 100. One ml. of diluted sol contains 50% of cholesterol, and 1 ml. of diluted plasma contains about 20% of cholesterol; about 40 per cent of the inhibitory power of plasma can therefore be attributed to the contained cholesterol, which is the same figure as that arrived at from other premises (Tsai and Lee, 1941, Ponder, 1944).

3. The results do not provide any indication that the action of any one of the three lysins on the red cell membrane is exclusively or even largely a reaction with a cholesterol component (as when digitonin forms a digitonide with cholesterol). The general inverse relation of the lytic activity and the cholesterol sol inhibition at different pH's is consistent with the cholesterol sol inhibition having scarcely any pH dependence of its own, the variations being simply the result of competition (see section 1 above); the activity of the lysin in its reaction with the material of the red cell membrane, on the other hand, may have a very pronounced pH dependence.

#### SUMMARY

The effects of variations of pH, from 5.6 to 8.6, on the lytic activity of saponin, digitonin, and sodium taurocholate, and the effects on the inhibition produced by plasma and by cholesterol sols, are described. The results, so far as they can be analyzed, show that the pH dependence of the inhibition produced by plasma is different from that of the inhibition produced by cholesterol sols, and this leads to the conclusion that a considerable part of the inhibition produced by plasma is due to inhibitory substances other than cholesterol. The results also provide a certain amount of indirect evidence that hemolysis is not due primarily to a reaction of the lysins with the cholesterol of the red cell membrane.

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# THE MECHANISM OF THE INHIBITION OF HEMOLYSIS

## III. INHIBITION BY SOLS OF SUBSTANCES RELATED TO CHOLESTEROL

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It has already been shown that the acceleration produced in hemolytic systems by substances such as the straight chain alcohols, the halogenated derivatives of benzene, etc., is closely related to their chemical structure (Ponder, 1939, Ponder and Hyman, 1939), and that even very small changes in structure result in changes in the acceleration observed (Ponder, 1941). This paper is concerned with the way in which the inhibitory power of cholesterol and certain related substances is dependent on molecular structure, and will show that small structural differences produce appreciable differences in the inhibition observed.

### *Material and Methods*

The cholesterol and its derivatives were kindly provided by Dr. Harry Sobotka, who had obtained them in a highly purified state for his own experiments on surface films. Each was dissolved in absolute methanol in the proportion of 5 mg. to 2 ml. of the alcohol, and 2 ml. of the resulting solution was then added to 20 ml. of warm water in order to make a sol by the method of Lee and Tsai (1942). The volume of the sol is reduced to about 9 ml. by gentle boiling, and after cooling is made up to 10 ml. with water. The resulting sols are quite stable, except in the case of *epi*-cholestanol, which tends to separate out when added to the warm water, and of cholestanol and cholesteryl acetate, which tend to flocculate when added to saline. It is best to prepare the sols shortly before each experiment, and to dilute them with saline (1 in 10, 1 in 20, etc.) immediately before they are used. When this is done, the results are quite reproducible.

In these sols, the cholesterol derivatives are in a micellar state, with a mean micellar diameter of about 0.1  $\mu$ . There is therefore considerable development of surface at the phase boundary between the particles of the sol and the phase containing the lysin, and the possibility has presented itself that some of the inhibitory effects observed may be due to lysin molecules becoming adsorbed at these surfaces in a non-specific manner. No inhibition of either saponin or digitonin hemolysis is obtained with sols of palmitic acid, stearic acid, or a variety of paraffin oils, all of which make excellent sols resembling the sols of the cholesterol derivatives so far as their dispersion goes. This answers the question of the possibility of a non-specific adsorption and consequent inhibition as well as it can be answered meantime.

*Hemolytic Systems.*—These are prepared by adding 0.8 ml. of diluted sol to 0.8 ml. of various dilutions of lysin, buffered at pH 6.5 with phosphate buffer. The mixtures are allowed to stand for 24 hours at 37°C. so that the reaction between lysin and in-



hibitor may reach virtual completion; 0.4 ml. of red cell suspension (the thrice washed cells of 1 ml. of human blood suspended in 20 ml. of saline) is then added, and the time for complete hemolysis observed. A standard time-dilution curve for a system containing 0.8 ml. of various solutions of lysin, 0.8 ml. of saline, and 0.4 ml. of cell suspension is plotted for each lysin. If a concentration of lysin  $c_1$  takes time  $t$  to produce complete hemolysis in the presence of a quantity  $Q$  of inhibitor, and if it is found by reference to the standard time-dilution curve that another (smaller) concentration  $c_2$  takes the same time to bring about complete hemolysis in the absence of the inhibitor, the inhibition produced by the quantity  $Q$  is  $\Delta = c_1 - c_2$ , which implies that the inhibitor reacts with the lysin  $c_1$  and renders inert an amount  $\Delta$  of it; the amount of lysin which remains is  $c_2$ . Alternatively, the inhibition can be measured by the ratio  $R = c_1/c_2 = c_1/(c_1 - \Delta)$ , the values of  $\Delta$  and  $R$  being easily convertible into one another for the same value of  $c_1$ .

### RESULTS

The results with sols containing 5  $\gamma$  of each of the derivatives are shown in Table I, and can be summarized as follows.

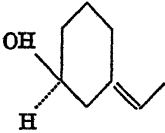
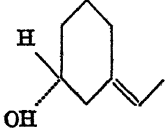
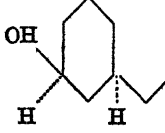
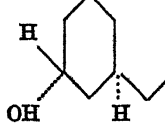
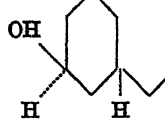
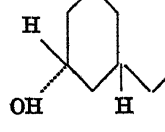
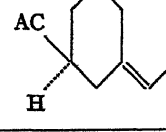
1. *Inhibition of Digitonin Hemolysis.*—The inhibition seems to depend on the arrangement of the H and OH atoms attached to carbons 3 and 5. In all cases the normal configuration has a greater inhibitory effect than the *epi* configuration, irrespective of whether there is a double bond at C5 or not. When a hydrogen on C5 replaces the double bond, some of the inhibitory power is lost, and the inhibition associated with the *trans* hydrogen is greater than that associated with the *cis* hydrogen. The combination of the *epi* configuration with the *cis* hydrogen on C5 (*epi*-coprosterol) results in the loss of all inhibitory power.

2. *Inhibition of Saponin Hemolysis.*—As in the case of digitonin, normal cholesterol is more inhibitory than its epimer. When the double bond at C5 is saturated, however, the *epi* configuration is *more* inhibitory than the normal form, the effect of a *trans* hydrogen being greater than that of a *cis* hydrogen, as in the case of the inhibition of hemolysis by digitonin.

3. *Relation of the Inhibitory Effect to the Windaus Reaction.*—In the case of the inhibition of digitonin hemolysis by cholesterol, there is a prevalent opinion that the reaction which is responsible is the same as that generally used for cholesterol determinations (Windaus reaction), and that it depends entirely on the normal configuration of the H and OH on C3. The evidence for this is more fragmentary than is usually realized. Hauseman (1905) concluded that both the H and OH on C3 and also the double bond at C5 are involved in the detoxifying action of cholesterol and related substances, engagement of the OH by esterification being found to destroy the detoxifying effect and saturation of the double bond to lessen it; detoxifying effects, however, cannot be measured with anything like the precision which is required in work with hemolytic systems. By methods of not much greater sensitivity, Abderhalden and LeCount (1906)

TABLE I

*Inhibition of Saponin ( $c_1 = 100 \gamma$ ) and Digitonin ( $c_1 = 80 \gamma$ ) by 5  $\gamma$  of Material in Sol, at 37°C., after Contact of Material and Lysin for 24 Hours at 37°C.*

Substance		Saponin in $\gamma$	Digitonin in $\gamma$
Cholesterol		27	63
<i>epi</i> -Cholesterol		9	35
Cholestanol		5	50
<i>epi</i> -Cholestanol		20	19
Coprosterol		0	29
<i>epi</i> -Coprosterol		9	0
Cholesteryl acetate*		7	10

\* Eastman Organic No. 2391, recrystallized.

also found that cholesterol, but not its esters, is inhibitory for saponin hemolysis, and recently Hsu and Yang (1941) have relied on these observations in discussing the relation of the inhibitory power of plasma to its content of total and free cholesterol. Windaus later found that normal cholestanol is inhibitory for digitonin hemolysis while *epi*-cholestanol is almost inert, but the "almost," and Hauseman's effect of saturating the double bond, seem to have been lost sight of, and it is often forgotten both that substances other than cholesterol can react with digitonin and saponin (see Bills, 1935, Bloor, 1943), and that the reactions may involve parts of the sterol molecule other than the OH of the Windaus reaction.

The results in Table I show that in the case of digitonin the principal inhibitory effect depends on the normal configuration of H and OH at C3, this allowing of an insoluble digitonide. Subsidiary inhibitory effects, presumably involving some sort of engagement of the molecule of the lysin with the molecule of the inhibitor, depend on the double bond at C5 or on the orientation of the H which replaces it.

In the case of saponin we have the unexpected result that the epimer is more inhibitory than the normal form when the double bond is saturated. This may be an expression of the fact that there are different ways in which substances like saponin and the sterols may react. The first is by forming compounds similar to the insoluble digitonides. The other is by the *epi* sterols forming liquid, expanding films which interpenetrate with saponin films in the same way as they do with films of digitonin (Langmuir, Schaefer, and Sobotka, 1937). This property may confer as much inhibitory power as the property of forming insoluble digitonide-like compounds.

Cholesteryl acetate has a small but distinct inhibitory effect on hemolysis by both saponin and digitonin. This appears to be another example of the subsidiary inhibitory effects already referred to.

#### SUMMARY

1. When digitonin is the lysin, the inhibitory power of sols of cholesterol and related substances depends primarily on a normal, as opposed to an *epi*, configuration of H and OH at C3. Subsidiary inhibitory effects depend on whether the double bond of cholesterol at C5 is saturated or not, and a *trans* hydrogen at C5 is associated with a greater inhibition than a *cis* hydrogen.

2. When saponin is the lysin, normal cholesterol is more inhibitory than its epimer. When the double bond at C5 is saturated (cholestanol, coprosterol, and their epimers), the *epi* configuration is more inhibitory than the normal configuration. This may be associated with the tendency of the epimers to form liquid interpenetrating films with films of digitonin and digitonin-like lysins.

3. At least one of the esters of cholesterol (cholesteryl acetate) has a small but definite inhibitory effect on both digitonin and saponin hemolysis.

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# STREPTOCOCCAL FIBRINOLYSIS: A PROTEOLYTIC REACTION DUE TO A SERUM ENZYME ACTIVATED BY STREPTOCOCCAL FIBRINOLYSIN\*

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## INTRODUCTION

Tillett and Garner (1933) observed that cultures or culture filtrates of certain strains of beta hemolytic streptococci bring about rapid lysis of the fibrin clot formed on adding calcium or thrombin to human plasma. This reaction they termed "fibrinolysis" and the active agent in the cultures, "fibrinolysin." Fibrinolysin has been found by other investigators to be associated most consistently with hemolytic streptococci of the Lancefield groups A, "human" C, and G (Tillett, 1938). While not peculiar to these streptococci, it is infrequently encountered in other species of bacteria.

Tillett and Garner (1933) observed in addition, that human plasma and human fibrinogen-thrombin clots are susceptible to the action of fibrinolysin, whereas plasma clots from rabbits are resistant. Other investigators have confirmed this observation and extended the studies to the fibrins of additional animal species (Tillett, 1938). LeMar and Gunderson (1940), in an extensive study of the susceptibility of fibrins from various species have shown that human fibrin is the most susceptible and that animal fibrins are usually partially or completely resistant. Resistance is not a property of the fibrin itself, since both Tillett and Garner (1933) and Madison (1934-35) have shown that resistant fibrin clots of animals become susceptible to streptococcal fibrinolysin if the animal fibrinogen is clotted with human thrombin. Similarly, if human fibrinogen is clotted with animal thrombin, these clots are also susceptible. It appears, therefore, that the resistance of animal clots is not due to insusceptibility of the fibrin itself, but to some other factor or factors.

Milstone (1941) has shown that if human fibrinogen and thrombin are purified by reprecipitation and exhaustive washing, the clots prepared from these materials will not lyse on the addition of an active culture or culture filtrate. If, however, human serum or the water-insoluble globulins from human serum are added to the system, the clots become normally susceptible to lysis. Furthermore, addition of human

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serum or the globulin fraction to rabbit plasma clots renders them as susceptible to fibrinolysis as are human plasma clots. Milstone concluded that human serum, and particularly the water-insoluble globulin of human serum, contains a component which he called "lysin factor," which is necessary, together with fibrinolysin, in order to produce the fibrinolytic phenomenon. According to Milstone, human fibrinogen-thrombin clots are susceptible to fibrinolysis because lysin factor is present as a contaminant in both these reagents; rabbit plasma clots are resistant because they lack a suitable lysin factor.

The nature of the chemical reaction or reactions associated with fibrinolysis has not been well understood. Garner and Tillett (1934) were unable to detect significant evidence of proteolysis during fibrinolysis. Jablonowitz (1937, 1939), however, has reported slight changes in the immunological specificity and salt-precipitability of fibrin following lysis, which could be interpreted as evidence of proteolysis.

It seemed probable that fibrinolysis results from proteolysis of the fibrin molecule and that failure of earlier investigators to detect significant digestion of fibrin might be due to the relatively low activity of the culture filtrates used as a source of fibrinolysin, to a low concentration of lysin factor in the system, or to a combination of these factors. In order to test these hypotheses, streptococcal fibrinolysin from large volumes of culture filtrate was concentrated and partially purified. Using this concentrated material, together with a concentrated lysin factor preparation, a study of the mechanism of the fibrinolytic reaction was undertaken in an effort to determine whether or not proteolysis of the substrate occurs, and also to investigate the nature of the interaction between fibrinolysin and lysin factor. The data which have been obtained indicate that fibrinolysis is incident to proteolysis of the fibrin molecule, and that proteins other than fibrin or fibrinogen can be digested. The data also indicate that lysin factor represents a proteolytic enzyme normally present in human serum, but in an inactive state. On the addition of streptococcal fibrinolysin, lysin factor is activated in a manner analogous to the activation of trypsinogen by enterokinase.

### *Materials and Methods*

1. *Choice of Streptococcal Strain for Fibrinolysin Production.*—A "human" group C strain of *Streptococcus hemolyticus* (H46A) was used for the production of fibrinolysin. This choice was influenced by the following factors: (a) of over 100 fibrinolytic strains tested, strain H46A produced the most active fibrinolytic filtrates, (b) this strain of group C *Streptococcus hemolyticus* does not produce scarlatinal (erythrogenic) toxin, and (c) strain H46A is not so fastidious in its growth requirements as the majority of group A strains, so that a defined medium can be prepared more easily.

2. *Medium for Production of Fibrinolysin.*—The obvious advantages of a protein-free medium of defined composition in attempts to isolate a metabolic product led to the adoption of the medium which Bernheimer, Gillman, Hottle, and Pappenheimer (1942) prepared for the cultivation of strain C203 of *Streptococcus hemolyticus* group

A. The principal modification made possible by the use of strain H46A was a 75 per cent reduction in the amount of glutamine added. Unfortunately, only certain lots of casein hydrolysate supported massive growth of the strain H46A. Investigation indicated that these lots of casein contained some factor necessary for massive growth, which was lacking in most lots of casein available. Because of this difficulty, Difco neopeptone was substituted for the acid hydrolysate of casein, since massive growth could be obtained uniformly under these conditions.

The following is the composition of the medium as used throughout most of the present studies:

Neopeptone base:	Neopeptone (Difco).....	150.00 gm.
	$\text{KH}_2\text{PO}_4$ .....	45.00 gm.
	Cystine in HCl.....	1.66 gm.
	Phenol red in alcohol.....	50.00 mg.
	Distilled water.....	2500.00 ml.

Adjust the mixture to pH 7.6 with 5N NaOH and heat to boiling. Filter through paper and autoclave at 121°C. for 20 minutes.

Eleven liters of distilled water in a 20 liter Pyrex carboy equipped with a mechanical stirring device are autoclaved for 1 to 2 hours and stored in the incubator until used. Immediately before inoculation, the 2½ liters of neopeptone base, together with the following reagents, are added to the carboy:

$\text{KHCO}_3$ crystals (autoclaved and dried in the incubator).....	30.00 gm.
Thioglycollic acid (Eastman technical grade).....	2.25 ml.
50 per cent Cerelose (technical grade dextrose).....	25.00 ml.
Addition mixture.....	150.00 ml.

The addition mixture has the following composition:

Uracil.....	150.0 mg.
Adenine sulfate.....	150.0 mg.
Biotin.....	15.0 micrograms
Nicotinic acid.....	15.0 mg.
Pyridoxine.....	22.0 mg.
Tryptophane.....	300.0 mg.
Calcium pantothenate.....	75.0 mg.
Thiamin chloride.....	37.0 mg.
Riboflavin.....	7.5 mg.
Glutamine.....	750.0 mg.
Salt mixture.....	30.0 ml.
Water to.....	150.0 ml.

The salt mixture is the one described by Bernheimer, Gillman, Hottle, and Pappenheimer (1942).

The carboy is inoculated with the contents of a lyophilized vial of strain H46A representing about 2 to 5 ml. of neopeptone-meat infusion broth culture, and incubated at 37°C. The low glucose concentration permits growth to become well established overnight without excessive production of acid. The following morning, 1 liter of 50 per cent Cerelose is added and incubation continued. The culture is maintained in the neutral range by addition of 5N NaOH at intervals of 15 to 20 minutes. The growth rate usually attained requires the addition of about 10 ml. of 5N base per



liter per hour. A growth rate which requires less than 4 ml. of base per liter per hour results in a concentration of fibrinolysin too low to be worth further processing.

It has been shown previously (Mueller and Klise, 1932; Bernheimer and Pappenheimer, 1942) that provided the medium is adequate in other respects, the chief factor limiting streptococcal growth is the large amount of acid produced. If the acid is continuously neutralized, growth continues until a second inhibiting factor comes into play—namely, the high concentration of sodium lactate formed upon neutralization of

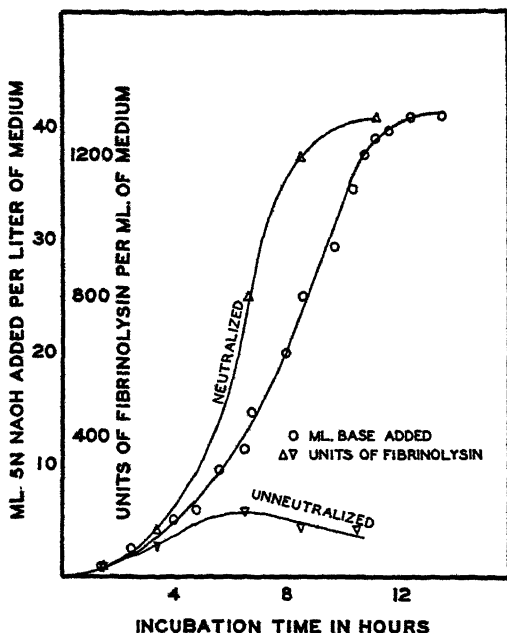


FIG. 1. Relation between acid production and fibrinolysin production in cultures of strain H46A, *Streptococcus hemolyticus*, group C. Acid production is expressed in terms of milliliters of 5N NaOH added to maintain neutrality of the culture. Fibrinolysin units are expressed as the reciprocal of the highest dilution lysing a standard fibrin clot in 30 minutes (Christensen, 1941).

the acid (Bernheimer and Pappenheimer, 1942). If the culture is not neutralized, not only are growth, acid production, and fibrinolysin production greatly diminished, but as time goes on, there is some destruction of preformed fibrinolysin. When the culture is kept neutral, however, fibrinolysin and acid production parallel each other, as shown in Fig. 1.

3. *Fibrinolysin Solutions*.—A 1:10 dilution of either the crude or partially purified concentrate was made up in gelatin buffer at pH 7.4. Experience has shown that this concentration is an appreciable excess in the usual test system.

4. *Lysin Factor Solutions*.—Milstone's (1941) procedure of diluting human serum with 19 volumes of distilled water containing 0.32 volume of 1 per cent acetic acid was

usually employed to precipitate the factor. Preparations of equal activity can be obtained by dialyzing the serum against distilled water and acidifying with acetic acid to pH 5.3. The most effective procedure when handling large volumes of serum was found to be precipitation of the factor by  $\frac{1}{2}$  saturation of the serum with ammonium sulfate. The precipitate was washed thoroughly with  $\frac{1}{2}$  saturated ammonium sulfate to remove other serum fractions carried down in the first precipitation. Whatever method of precipitation was used, the precipitate was collected by centrifugation and dissolved in a volume of saline buffer at pH 7.4 equal to  $\frac{1}{10}$ th the original serum volume.

5. *Fibrinogen*.—This was prepared by salting out with ammonium sulfate or sodium chloride and preserved by lyophilization as described in previous reports (Christensen and Jones, 1939, Christensen, 1940). Solutions of the desired concentration were made up in saline buffer, pH 7.4.

6. *Thrombin*.—Earlier, thrombin prepared from human plasma by the method of Tillett and Garner (1933) was used. Later, a commercial preparation of rabbit thrombin, hemostatic globulin,<sup>1</sup> was routinely employed in a dilution of 1:25 in saline buffer.

7. *Casein*.—Casein was prepared from fresh skim milk by acid precipitation followed by dehydration with alcohol and ether, according to the method described by Hawk and Bergeim (1937). For use, the dry powder was suspended in saline buffer and dissolved with the aid of alkali to make a 5 per cent solution at pH 7.4.

8. *Gelatin*.—Eastman purified calfskin gelatin was dissolved in saline buffer with the aid of heat to make a 4 per cent or 5 per cent solution at pH 7.4.

9. *Saline Buffer*.—M/10  $\text{KH}_2\text{PO}_4$  was adjusted to pH 7.4 with 5N base before making up to volume. The solution contained 0.9 per cent sodium chloride.

10. *Gelatin Buffer*.—Saline buffer containing 0.5 per cent Eastman purified calfskin gelatin, pH 7.4.

11. *Acetate Buffer*.—M/5 sodium acetate adjusted to pH 3.5 with M/5 acetic acid. pH determinations on all solutions were carried out electrometrically, using a glass electrode.

All solutions containing organic reagents were preserved by the addition of 1:10,000 merthiolate. Madison and Snow's (1937) observation that merthiolate is without effect on the fibrinolysin reaction has been confirmed in this laboratory.

### *Determination of Lysin Factor-Fibrinolysin Activity*

1. *Fibrinolysis Time*.—The technique used was essentially that of Tillett and Garner (1933), with minor modification of some details. The test contained 0.2 ml. of plasma or a solution containing an equivalent amount of fibrinogen, 0.1 ml. of fibrinolysin solution, and 0.2 ml. of rabbit thrombin (hemostatic globulin). Before addition of the thrombin solution, the volume was made up to 0.8 ml. with saline buffer to which was added any other reagents to be included in the test. After addition of the thrombin solution, the clots form within 1 minute. The tubes were incubated in a water bath at 37°C. Lysis time was taken as the time between formation and complete disappearance of the fibrin clot.

2. *Decrease in Acid-Precipitable Nitrogen*.—Aliquots of the lysin factor-fibrinolysin-

<sup>1</sup> The hemostatic globulin was kindly supplied by the Lederle Laboratories, Inc.

substrate mixture, consisting of 0.5 ml. of fibrinolysin solution, 0.5 ml. of lysin factor solution, and 6.0 ml. of substrate were blown into an equal volume of 10 per cent trichloroacetic acid in Pyrex test tubes with conical tips. The mixture was allowed to stand for 15 to 30 minutes to ensure complete precipitation and the precipitates were packed by centrifugation. After washing with 5 per cent trichloroacetic acid, the precipitates were digested in the same tubes and the nitrogen content determined.

3. *Increase in Acid-Soluble "Tyrosine"*.—Anson and Mirsky (1932, 1933, 1937) have used a modification of Folin and Ciocalteu's (1927) phenol method to measure proteolysis. In this method, the acid-soluble tyrosine and tryptophane-containing split products of protein digestion are measured and expressed as liberated tyrosine. Recently, Heidelberger and MacPherson (1943) have improved the method by substituting sodium carbonate for sodium hydroxide and employing a Coleman spectrophotometer to measure the color developed. Based on these modifications, the analysis was carried out as follows: Aliquots of the reaction mixture were precipitated with an equal volume of 10 per cent trichloroacetic acid. After flocculation, the precipitate was removed by filtration. 2.0 ml. of the filtrate were placed in a Pyrex tube, 6.0 ml. of 12.5 per cent sodium carbonate added, and the contents of the tube mixed. 1 ml. of the Folin and Ciocalteu phenol reagent, diluted 1:3, was added with shaking and the color allowed to develop for about 30 minutes. The color was read in a Coleman spectrophotometer at 650 m $\mu$  with the reagent blank set at 100 per cent transmittance. "Tyrosine" values were calculated from a calibration curve prepared with pure tyrosine.

4. *Liberation of Amino Nitrogen*.—Aliquots of the reaction mixture, usually 5 to 10 ml., were placed in a small beaker and adjusted to pH 7.0 with acid or alkali. Neutral formaldehyde (Merck reagent) was added so that the final concentration after titration would be between 6 and 9 per cent and the solution was titrated to pH 9.1 with 0.02N alkali, as recommended by Levy (1934). Titration was carried out electrometrically, using a Beckman electrometer with shielded glass electrode.

5. *Decrease in Viscosity*.—Equal volumes of fibrinolysin solution and lysin factor were mixed and incubated for 10 minutes to attain temperature equilibrium and allow activation of the lysin factor. 6 ml. of gelatin or other protein solution, warmed in the water bath to 37°C. were mixed with 1 ml. of the lysin factor-fibrinolysin solution and immediately poured into the pipette. Ostwald viscosity pipettes, with a flow time of between 15 and 24 seconds with distilled water at 37°C., were used. The water bath was maintained at  $37 \pm 0.1^\circ\text{C}$ . The time of mixing the lysin factor-fibrinolysin mixture and the substrate solution was noted and the flow time of the reaction mixture determined at intervals of a few minutes, usually for a total period not exceeding 60 minutes.

6. *Quantitative Determination of Proteolytic Activity*.—The procedure used by Northrop (1932-33) to quantitate pepsin activity in terms of the per cent change in the specific viscosity of gelatin was adopted with slight modification. Northrop's equation for the calculation of pepsin units is as follows:  $\frac{\Delta V}{V_0 \Delta T} = \text{pepsin units}$ , where  $V$  is the specific viscosity and is equal to  $\frac{ts}{t_{\text{H}_2\text{O}}} - 1$ , when  $ts$  is the flow time of the reaction mixture and  $t_{\text{H}_2\text{O}}$  is the flow time of distilled water in the pipette.

With the lysin factor-fibrinolysin system the reaction was carried out at pH 7.4 with 6 ml. of 4 per cent gelatin as the substrate and 1 ml. of the lysin factor-fibrinolysin mixture. The specific viscosity values obtained were plotted and a line drawn through the points. Activity was expressed in terms of proteolytic units (p.u.) and was calculated as  $\frac{(V_0 - V_{10}) 100}{V_0 \times 10}$  where  $V_0$  is the initial specific viscosity and  $V_{10}$  is the viscosity at 10 minutes.

#### EXPERIMENTAL

##### *Concentration and Partial Purification of Fibrinolysin*

Fifteen or 30 liter lots of cultures of strain H46A, group C *Streptococcus hemolyticus* were cultivated by the methods described above. After growth had ceased, due to exhaustion of the glucose, the streptococcal cells were removed in a Sharples centrifuge. The supernatant, adjusted to pH 4.5 with glacial acetic or concentrated hydrochloric acid, was stored in the refrigerator. The following day the supernatant was concentrated to about  $\frac{1}{10}$ th the original volume in a large vacuum still at about 25°C. The syrupy concentrate was saturated with solid ammonium sulfate and allowed to stand at room temperature until flocculation was complete. A dark brown, flocculent precipitate formed and was collected on No. 50 Whatman paper by suction filtration. The precipitate, which contained practically all of the fibrinolytic activity of the original culture, was dissolved in saline buffer at pH 7.4 and centrifuged at high speed in a Swedish angle centrifuge to remove as completely as possible the cells and debris remaining after the original Sharples centrifugation. Since some fibrinolysin appeared to be adsorbed on the precipitate, the sedimented material was extracted 2 to 3 times with saline buffer and the extracts combined with the supernatant. The usual practice has been to maintain the active material as a precipitate in saturated ammonium sulfate solution in a volume representing a 150- to 200-fold concentration of the culture. In this form fibrinolysin is stable. Several preparations have shown no loss of activity in over 2 years when stored in the refrigerator.

Further purification can be accomplished by taking advantage of the fact that fibrinolysin is insoluble at a pH of 4.5 or below, while much inactive material is soluble. The crude fibrinolysin precipitate, prepared as described above, was dissolved in saline buffer with the aid of alkali in a concentration about 100 times that of the original culture. When acidified below pH 4.5 with concentrated acid, an abundant flocculent precipitate formed. This precipitate was washed with 200 ml. portions of acetate buffer, pH 3.5, until the washings were colorless. This procedure removed a considerable amount of color, derived mainly from the neopeptone in the medium, most of the residual phenol red, together with a considerable amount of inert, acid-soluble, nitrogenous material. The acid-extracted precipitate was dissolved in 100 ml. saline buffer at pH 7.4. The solution was dark brown in color, and still contained some insoluble material, chiefly bacterial cells, which could not be removed at this point by centrifugation or filtration because of the high viscosity of the solution. However, when this viscous solution was added to 5 to 10 volumes of alcohol, and the resulting precipitate redissolved in 100 ml. of saline buffer, the viscosity was reduced to such a degree that the insoluble debris could be spun out, leaving a dark brown, clear solution.

The insoluble precipitate was extracted twice with 100 ml. portions of saline buffer to elute adsorbed fibrinolysin, the extracts and supernatant pooled and precipitated by acidification. The precipitate was dissolved in saline buffer at pH 7.4 in a concentration 100 times that of the original culture. The nitrogen content of the solution was equivalent to a protein concentration of about 1 per cent, and represented about 4 per cent of the total nitrogen of the original culture medium. A flow chart of a typical preparation, with the nitrogen values of the material at the various stages is shown in Table I.

As indicated above, the fibrinolysin concentrate could not be freed from color by acid extraction nor could the color remaining after acid extraction be separated from the fibrinolysin by treatment with Norit charcoal, alumina gel, or by dialysis.

The partially purified material can be salted out by ammonium sulfate at concentrations between 22 and 40 per cent of saturation. Attempts to fractionate between these concentrations have resulted in smearing of the activity over the various fractions.

The fibrinolytic activity of the concentrated material, prepared in the manner just described, is only slowly destroyed by heating at 100°C at pH 7.4. It is stable at refrigerator temperature over a pH range of 3.0-8.5. It is readily destroyed, however, by treatment with trypsin and pepsin, indicating that the activity is associated with a protein.

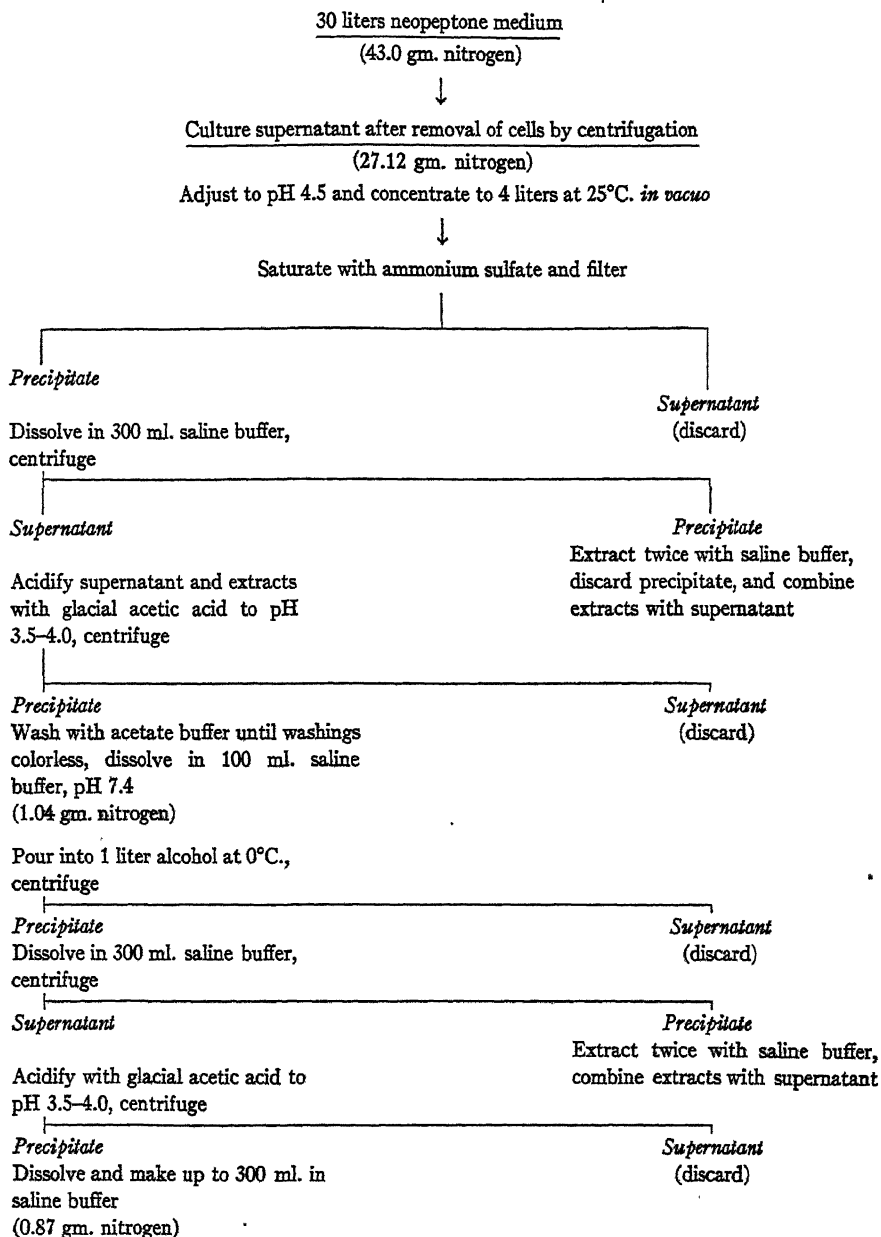
#### *Proteolytic Nature of Fibrinolysis*

Twenty-five ml. of a solution of human fibrinogen precipitated by sodium chloride and equivalent to the fibrinogen in an equal volume of plasma were placed in a flask to which was added 20 ml. of a fibrinolysin concentrate which had been dialyzed ammonium sulfate-free, together with 5 ml. of a human thrombin solution. A second flask was prepared in a similar manner, except that saline buffer was substituted for thrombin. The flasks were incubated in a water bath at 37°C. and at intervals, 5 ml. portions of the contents of both flasks were removed and the trichloroacetic acid-precipitable nitrogen determined. Zero time values were obtained by adding the reagents separately to the acid in order to avoid proteolysis.

From the data obtained in this experiment, as shown in Fig. 2, it is evident that the action of fibrinolysin on human fibrin and fibrinogen results in an appreciable decrease in acid-precipitable nitrogen. Furthermore, under the conditions of the experiment, there appears to be no significant difference between the hydrolysis of fibrin and fibrinogen.

It was felt desirable to confirm the evidence of proteolysis by other means. Since no differences could be detected between the hydrolysis of fibrin and fibrinogen, fibrinogen was used thereafter as a substrate in most experiments because the addition of another protein, thrombin, was unnecessary; and the substrate remained in solution throughout the experiment. Using a reac-

TABLE I

*Concentration and Partial Purification of Streptococcal Fibrinolysin*

tion mixture consisting of 12 volumes of a 5 per cent fibrinogen solution with 1 volume of lysin factor activated by 1 volume of fibrinolysin solution, the proteolytic nature of the reaction was confirmed by demonstrating the liberation of amino nitrogen, increase in acid-soluble "tryrosine," and decrease in the viscosity of the mixture, as shown in Fig. 3. If lysin factor is not added to the reaction mixture, proteolysis occurs but to a lesser extent. This is due in all likelihood to the presence of lysin factor in the fibrinogen solution, since as stated above, no attempt was made to prepare the fibrinogen free from lysin factor.

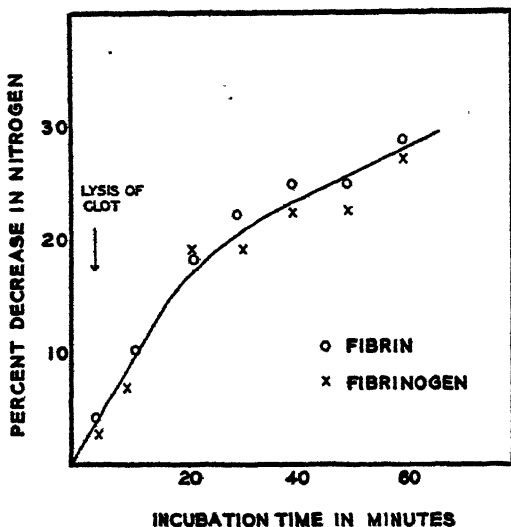


FIG. 2. Decrease in acid-precipitable fibrin and fibrinogen nitrogen following treatment with fibrinolysin. Results are expressed as per cent decrease in the nitrogen found in the precipitate.

#### *Rôle of Lysin Factor*

With definite evidence that fibrinolysis and fibrinogenolysis involve proteolysis of the substrate, it became of interest to ascertain the rôle of lysin factor in the reaction. Milstone (1941) has shown that both fibrinogen and thrombin, as ordinarily prepared, are contaminated with lysin factor. Clots prepared from fibrinogen and thrombin purified by Milstone's method usually do not lyse when treated with unconcentrated culture filtrates as a source of fibrinolysin. However, when these clots are treated with concentrated fibrinolysin, lysis usually occurs. Because of the labor involved in purifying fibrinogen, and because it proved extremely difficult to prepare a product which would not lyse in the presence of highly concentrated preparations of fibrinolysin, other substrates were sought which would be free of lysin factor.

1. *Lysis of Rabbit Fibrin*.—Milstone (1941) found that rabbit plasma clots do not lyse on the addition of active culture filtrates unless human lysin factor is also added. These results were interpreted by Milstone as indicating the lack of a suitable lysin factor in rabbit serum. When attempts were made to repeat these experiments with concentrated fibrinolysin, it became evident that the difference between human and rabbit plasma clots is not due to lack of lysin factor in the latter. With concentrated fibrinolysin, rabbit plasma clots

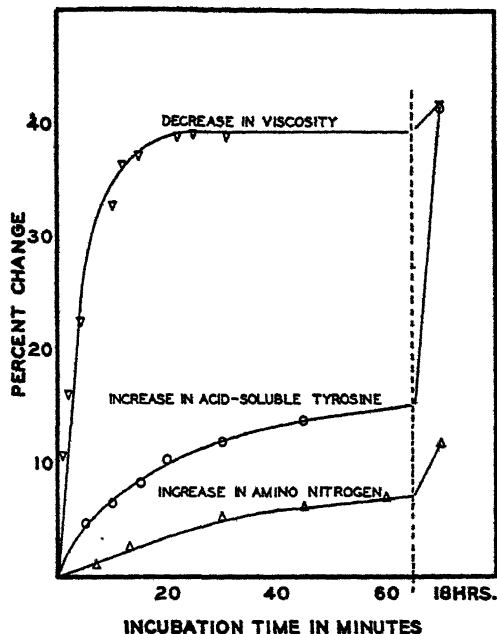


FIG. 3. Proteolysis of fibrinogen by lysin factor-fibrinolysin. The decrease in viscosity is expressed as the per cent decrease from the initial relative viscosity; acid-soluble "tyrosine" and amino nitrogen are expressed as the per cent of total substrate "tyrosine" and nitrogen respectively.

undergo lysis readily, whereas on dilution of the fibrinolysin to approximately the concentration of the average culture filtrate, the clots do not lyse unless human lysin factor is added. The following experiment illustrates this finding. Duplicate sets of standard fibrinolysin tests were prepared with various dilutions of a fibrinolysin concentrate. Two-tenths ml. of human lysin factor solution was added to one series, an equal volume of saline buffer to the other. The tubes were placed in the water bath and the lysis time of the clots in the presence and absence of lysin factor noted. The data obtained are shown in Table II.

It is evident from the results shown in Table II, that while dilute solutions of



fibrinolysin do not cause lysis of rabbit plasma clots in the absence of added lysin factor, fibrinolysis takes place when concentrated fibrinolysin solutions are used.

The resistance of rabbit fibrin may be due to the presence of an inhibitor, in addition to a deficiency of lysin factor, as suggested by Milstone (1941). Evidence supporting this view was obtained in experiments which showed that addition of rabbit serum to human fibrin clots causes a prolongation of lysis time. As shown in Table III, when varying amounts of rabbit serum are added to the test system containing human fibrinogen clotted with thrombin in the presence of a constant amount of fibrinolysin, the higher concentrations of

TABLE II  
*Lysis of Rabbit Fibrin with Concentrated Fibrinolysin*

Dilution of fibrinolysin	Lysis time	
	With lysin factor	Without lysin factor
	<i>min.</i>	<i>min.</i>
Undiluted	1.5	10.0
1:10	1.5	16.5
1:40	1.5	Overnight*
1:160	3.5	No lysis†
1:640	14.0	" "
1:2560	Overnight	" "
Control	Negative	" "

\* Indicates no lysis observed during the first day's observation. Clots found lysed the following morning.

† No lysis observed in 24 hours.

rabbit serum produce a significant prolongation of the time required for fibrinolysis.

The presence of an inhibitor in rabbit plasma is also indicated by the following observations. Fibrinogen was isolated from a sample of rabbit plasma by salting out with sodium sulfate (fibrinogen I). A portion of this fibrinogen was further purified by a second precipitation with sodium sulfate (fibrinogen II). The two preparations were dissolved in saline buffer in a volume equal to the volume of the plasma from which they were isolated. Fibrinolysin tests were set up with these preparations, each containing, in addition to the fibrinogen or plasma, 0.2 ml. human lysin factor, 0.1 ml. of the fibrinolysin dilution, and 0.2 ml. of hemostatic globulin, diluted 1:25. The results of these tests are shown in Table IV.

As may be seen from the data presented in Table IV, purification of rabbit fibrinogen by reprecipitation results in a decrease in the lysis time of fibrin clots prepared from it, suggesting the removal of an inhibitor. This is most apparent in the tests in which the two highest dilutions of fibrinolysin were used.

2. *Proteolysis of Casein*.—This protein, similar to fibrinogen in that it is clotted by a specific enzyme, was tested as a possible substrate. One ml. of skim milk was clotted by rennin in the presence of fibrinolysin alone, lysin factor alone, and fibrinolysin and lysin factor together. The solutions were placed in a water bath at 37°C. and examined at intervals for evidence of lysis. The results are shown in Table V.

TABLE III  
*Inhibition of Lysis of Human Fibrinogen by Rabbit Serum*

Rabbit serum dilution	Lysis time of clots
	<i>min.</i>
Undiluted	120.0
1:10	6.0
1:100	4.5
1:1000	4.5
Control	4.5

Dilutions of rabbit serum in a volume of 0.1 ml. were added to the fibrinolysin test containing human fibrinogen clotted by hemostatic globulin in the presence of 0.1 ml. of fibrinolysin concentrate diluted 1:10.

TABLE IV  
*Effect of Fibrinogen Purification on Lysis Time of Rabbit Fibrin*

Fibrinolysin dilution	Lysis time of clots prepared from		
	Whole plasma	Fibrinogen I	Fibrinogen II
Undiluted	—	No clot*	No clot
1:10	1.5 min.	" "	1.5 min.
1:40	1.5 "	1.5 min.	1.5 "
1:160	3.5 "	5.5 "	3.5 "
1:640	14.0 "	16.0 "	8.0 "
1:2560	Overnight†	67.0 "	15.5 "

\* A fibrin clot never formed. This was due to destruction of the fibrinogen before clotting could occur, and is not infrequently seen with highly active fibrinolysin preparations.

† No lysis observed during the first day, clots found lysed the next morning.

As shown in Table V, neither fibrinolysin nor lysin factor alone had any effect on the casein clot. However, when both were present, "caseinolysis" analogous to fibrinolysis, occurred.

With evidence that casein is susceptible to the action of the lysin factor-fibrinolysin system, experiments similar to those with fibrinogen were carried out to determine the extent of proteolysis.

One volume of fibrinolysin solution and 1 volume of lysin factor were mixed and incubated for 10 minutes at 37°C. Twelve volumes of 5 per cent casein

solution were then added, mixed thoroughly, and incubation at 37° continued. Determination of acid-soluble "tyrosine," amino nitrogen, and viscosity of the

TABLE V  
"Caseinolysis" by Lysin Factor-Fibrinolysin

Skim milk	Lysin factor	Fibrinolysin	Rennin	Lysis time
ml.	ml.	ml.	ml.	min.
1.0	0.5	0.5	0.1	30-60
1.0	0.5	—	0.1	No lysis
1.0	—	0.5	0.1	" "

Milk was freed of fat by centrifugation. The rennin solution was prepared by dissolving one Chr. Hansen junket tablet in 10 ml. distilled water. The fibrinolysin concentrate was diluted 1:10 in saline buffer.

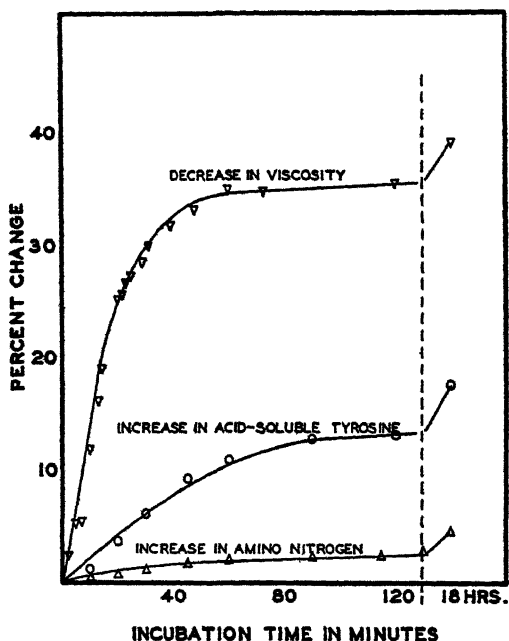


FIG. 4. Proteolysis of casein by lysin factor-fibrinolysin. Experimental details identical with those of Fig. 3, except that the substrate was 5 per cent casein instead of fibrinogen.

mixture at various time intervals showed that casein is susceptible to the proteolytic action of the lysin factor-fibrinolysin system. The results of this experiment are shown in Fig. 4.

3. *Proteolysis of Gelatin*.—A 5 per cent gelatin solution was tested for susceptibility to the action of the lysin factor-fibrinolysin system under the same

experimental conditions as in the case of fibrinogen and casein. Liberation of amino nitrogen and decrease in the viscosity of the mixture, as shown in Fig. 5, indicate that gelatin is also hydrolyzed.

The experiments with casein and gelatin support Milstone's (1941) hypothesis that both fibrinolysin and lysin factor must be present to constitute an active system. No hydrolysis of either casein or gelatin could be detected in the present experiments when either fibrinolysin or lysin factor was used alone, but only when both reagents were present.

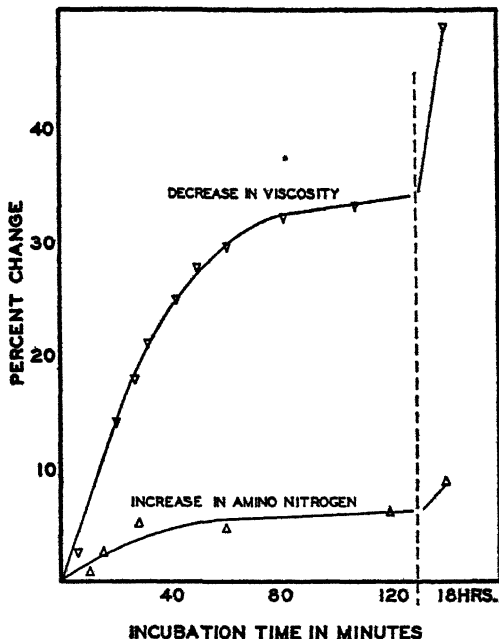


FIG. 5. Proteolysis of gelatin by lysin factor-fibrinolysin. Experimental details identical with those of Figs. 3 and 4, except for the substitution of gelatin as substrate.

#### *Nature of the Interaction between Lysin Factor and Fibrinolysin*

Since both fibrinolysin and lysin factor are necessary for activity, two possibilities may be suggested to explain their interaction. On the one hand, lysin factor may activate fibrinolysin, the latter being the proteolytic enzyme, or alternatively, fibrinolysin activates lysin factor and renders it proteolytic. The experiments to be detailed below indicate that in all probability lysin factor is the proteolytic agent which is activated by fibrinolysin.

1. *Spontaneous Activation of Lysin Factor.*—Samples of lysin factor which have been stored in the cold, after a variable period may develop proteolytic activity spontaneously. The proteolytic activity of a number of batches of

lysin factor was determined shortly after preparation and at intervals thereafter, both in the presence and absence of fibrinolysin. The activity of four of these samples, prepared by both dilution and acidification and by salting out, is shown in Table VI.

From the data presented in Table VI, it can be seen that in all instances, the addition of fibrinolysin to lysin factor which had become active spontaneously did not result in the development of more proteolytic activity than when fibrinolysin was added to the lysin factor before the latter had developed spontaneous activity.

If spontaneous activity of the aged lysin factor preparations were due to the presence of another proteolytic enzyme, it might be expected that the total

TABLE VI  
*Spontaneous Activation of Lysin Factor*

Lysin factor No.	Date tested	Activity in proteolytic units	
		With fibrinolysin	Without fibrinolysin
253	5/26/43	0.7	0
	6/2/43	0.7	0
	8/2/43	0.7	0.6
406	9/16/43	1.3	0
	11/3/43	0.7	0.6
513	2/12/44	2.4	0
	6/9/44	2.6	2.3
539	5/27/44	1.7	0
	6/9/44	1.7	1.8

Activity expressed in terms of proteolytic units in 0.5 ml. of lysin factor solution, determined by the procedure described in the section on Methods.

activity of the aged preparation, when treated with fibrinolysin would be equal to the sum of the original lysin factor-fibrinolysin activity plus the activity developed on ageing, but in no instance has such an increase in the total activity of the system been noted.

On the other hand, of numerous samples of fibrinolysin, none has ever shown any proteolytic activity in the absence of lysin factor, when tested on the lysin factor-free substrates, casein and gelatin.

*2. Catalytic Nature of Lysin Factor Activation.*—When serial dilutions of lysin factor are mixed with an excess of fibrinolysin and incubated, maximum proteolytic activity develops in less than 10 minutes, and is proportional to the concentration of lysin factor. The results of such an experiment are shown in Table VII.

On the other hand, when dilutions of fibrinolysin are mixed with constant amounts of lysin factor and incubated, the activity developed is proportional

not to the concentration of fibrinolysin, but to the incubation time of the lysin factor-fibrinolysin mixture. In other words, the total proteolytic activity

TABLE VII  
*Relation between Lysin Factor Concentration and Activity*

Lysin factor dilution	Activity in proteolytic units
Undiluted	3.1
1:2	1.8
1:4	0.7
1:6	0.6
1:8	0.4

Lysin factor solution in a volume of 0.5 ml. was activated by treatment with 0.5 ml. of 1:10 dilution of concentrated fibrinolysin for 10 minutes at 37° C. The activated lysin factor-fibrinolysin mixture was then mixed with 6 ml. gelatin, warmed to 37°, and the activity determined.

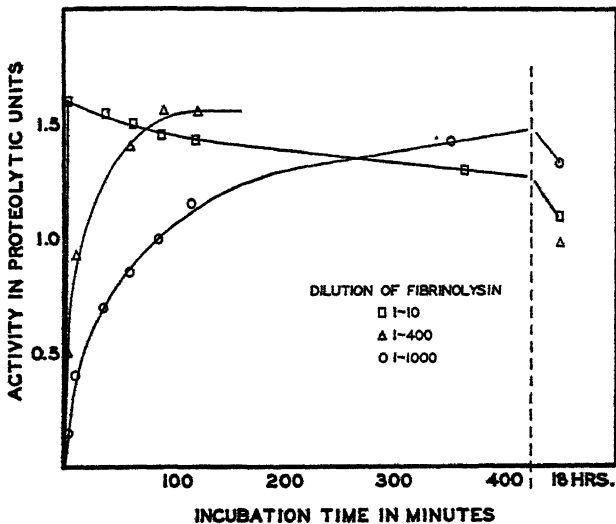


FIG. 6. Activation of lysin factor by fibrinolysin. Aliquots of lysin factor solution mixed with an equal volume of fibrinolysin dilutions and incubated at 25°C. At intervals, 1 ml. of each mixture removed and tested for activity by the procedure described under Methods.

developed with a constant amount of lysin factor and different amounts of fibrinolysin ultimately reaches the same level in each case, given a sufficiently long incubation period, as shown in Fig. 6. Furthermore, with a constant amount of lysin factor, the rate of activation is proportional to the fibrinolysin concentration.

These experiments indicate that lysin factor is activated by fibrinolysin and also that the activation is not due to a stoichiometric reaction between the two, but is catalytic in nature.

#### DISCUSSION

It is evident from the results of the experiments described in this paper that streptococcal fibrinolysis is a proteolytic reaction, the active proteolytic enzyme being produced as the result of an interaction of streptococcal fibrinolysin and the lysin factor normally present in plasma and serum. The dissolution of the fibrin gel, heretofore considered the characteristic feature of the fibrinolytic reaction, appears to be merely an early manifestation of proteolysis. It has also been shown that proteolysis is not limited to fibrin and fibrinogen, since in addition to these substrates, casein and gelatin are also susceptible to the action of the lysin factor-fibrinolysin system. Failure on the part of earlier workers to demonstrate an action of streptococcal fibrinolysin on proteins other than fibrin and fibrinogen was undoubtedly due to the fact that the part played by the serum component, lysin factor, was not recognized until the work of Milstone.

The essential rôle of lysin factor in the proteolytic reaction is most clearly demonstrated in the experiments with proteins other than fibrin and fibrinogen. The results of experiments with fibrinogen and thrombin must always be interpreted with caution because of the great difficulty in removing all traces of lysin factor from these reagents. As indicated in the experimental section, fibrinogen can be prepared sufficiently free from lysin factor that dilute solutions of fibrinolysin will not lyse fibrin clots prepared from it. Lysis of such fibrin clots on the addition of highly concentrated fibrinolysin might be interpreted as being due to a proteolytic action of the fibrinolysin itself. When highly concentrated fibrinolysin is mixed with other proteins, however, for example casein and gelatin, no trace of proteolysis has ever been detected unless lysin factor was also added. It appears, therefore, that the interaction of fibrinolysin and lysin factor is necessary for the production of the proteolytic agent, and that the slight susceptibility of purified fibrin clots to fibrinolysin is due to the presence of small amounts of lysin factor not removed during purification.

It has been assumed that the relative or absolute resistance of animal fibrins to streptococcal fibrinolysin is due to species differences in the fibrin itself. Tillett and Garner (1933) and Madison (1934-35), however, showed that "hybrid" fibrins are susceptible if one of the components, either fibrinogen or thrombin, is of human origin. These results were interpreted by Milstone (1941) as indicating a deficiency of a suitable lysin factor in the plasma of the resistant species, a lack which might be supplied by the human component of the system. Actually, as shown by the present data, rabbit fibrin clots are susceptible to lysis if sufficient fibrinolysin is used in the system. Additional evidence

that rabbit plasma contains lysin factor is indicated by the observation that successive precipitations of rabbit fibrinogen render the fibrin clots progressively more susceptible to the action of fibrinolysin.

The anomalous behavior of rabbit plasma as compared with human may be due to several factors. The present experiments indicate that rabbit serum contains an inhibitor of the proteolytic enzyme, which may account in part for the resistance of rabbit plasma clots. However, the presence of an inhibitor in these clots does not explain fully the susceptibility of "hybrid" clots. It is also possible therefore, that rabbit serum contains less lysin factor than does human, or that lysin factor in rabbit serum is qualitatively different and is activated by fibrinolysin at a slower rate than human lysin factor.

A plausible explanation for the necessity of interaction between lysin factor and fibrinolysin before the appearance of proteolytic activity is that one of these components activates the other. In the presence of excess fibrinolysin, the proteolytic activity developed is directly proportional to the concentration of lysin factor in the system, hence it appears that lysin factor is the component which is converted to a proteolytic enzyme. The validity of this hypothesis is also borne out by the fact that lysin factor preparations may develop proteolytic activity spontaneously, whereas in our experience fibrinolysin solutions never do. In addition, treatment of a constant amount of lysin factor with varying amounts of fibrinolysin results in the eventual liberation of an equal amount of proteolytic activity in each instance, and the rate of activation is proportional to the fibrinolysin concentration. It may be suggested, therefore, that lysin factor represents an inactive proteolytic enzyme or "zymogen" of serum which is activated by fibrinolysin. The analogy between this system and the trypsinogen-enterokinase and chymotrypsinogen-trypsin systems is striking. The lysin factor system is more nearly analogous to the chymotrypsinogen system, however, in that activation does not appear to be rapidly autocatalytic as is the case with the trypsinogen-trypsin system. It is not suggested, however, that lysin factor is chymotrypsinogen, since the two have different pH optima and differ in other respects.

Activation of a proteolytic enzyme from an animal source by a kinase derived from a microorganism is not without precedent, since Kunitz (1938) has shown that trypsinogen may be activated by a kinase obtained from cultures of a mold of the genus *Penicillium*.

It has been known for many years that human and animal sera contain a proteolytic enzyme normally present in an inactive state. In a recent series of papers, Tagnon and coworkers (1942) have reviewed much of the literature concerning this enzyme. Certain similarities between lysin factor and the serum protease are apparent. For example, both are found in the globulin fraction of serum; both are active at neutrality; both digest casein and gelatin as well as fibrin; and both are present in serum in an inactive state. As originally shown



by Nolf (1908), the serum protease is activated by treatment of the serum with chloroform. Lysin factor, on the other hand, is activated by streptococcal fibrinolysin. It is possible, however, that this difference merely represents two methods of activating the same enzyme.

Based on the evidence presented in this paper, the following hypothesis of streptococcal fibrinolysis is advanced. Lysin factor is an inactive proteolytic enzyme or "zymogen" in serum which is activated by a kinase, streptococcal fibrinolysin. Addition of fibrinolysin to a solution containing lysin factor results in the catalytic conversion of the lysin factor to an active proteolytic enzyme, which is able to cause fibrinolysis of fibrin clots or proteolysis of other proteins such as casein or gelatin. Demonstration of the action of streptococcal fibrinolysin and lysin factor on substrates other than fibrin or fibrinogen makes it obvious that the specific designation "fibrinolysin" for this component of streptococcal culture filtrates is inaccurate.

#### SUMMARY AND CONCLUSIONS

1. Methods for the preparation and partial purification of streptococcal fibrinolysin are described.

2. The lysis of fibrin clots in the presence of streptococcal fibrinolysin is associated with proteolysis of the fibrin. Digestion is due to an enzyme normally present in serum or plasma in an inactive state, which is activated by fibrinolysin. Fibrinolysin alone has no demonstrable proteolytic activity.

3. The lysin factor-fibrinolysin system brings about proteolysis of other proteins such as gelatin or casein, in addition to fibrin and fibrinogen.

4. It is suggested that lysin factor exists in serum or plasma as a zymogen, and that it is activated by fibrinolysin, a kinase, in a manner similar to the activation of trypsinogen by enterokinase or the mold kinase of Kunitz (1938).

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# MAXIMA IN RATE-CONCENTRATION CURVES AND THEIR RELATION TO THE STRUCTURAL ASPECTS OF CELLULAR METABOLISM\*

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## INTRODUCTION

In the course of an investigation into hydranth regeneration of the coelenterate *Tubularia*, it was noted (Moog and Spiegelman, 1942; Spiegelman and Moog, 1944) that some inhibitory reagents could, at certain concentrations, stimulate regenerative activity. A more systematic study was made of this phenomenon with two inhibitors, ethylurethane and phenylurethane. The relative simplicity of the biological system employed permitted quantitative measurements of regeneration rates. The results, which will be reported here, indicate that the stimulation is reproducible and is confined to a relatively small concentration range for a particular reagent.

This peculiar effect of inhibitors is not unique to regeneration but on the contrary is quite widespread in its occurrence. During the investigations of the pharmacological activity of various drugs it was often noticed that many of them were diphasic in their effect on physiological processes, stimulating them at one concentration and inhibiting them at another. This often made its appearance in terms of maxima in the rate-concentration plots. Starting at 100 per cent of normal for zero concentration, the curve would rise for very low concentrations of the reagent above this level, and then start to fall to values below the normal for higher concentrations of the drug. So widespread was this phenomenon that it led to the postulation of the so called "Arndt-Schulz law," which states that drugs which inhibit at high concentrations stimulate at low concentrations.

The same phenomenon was observed in the study of enzyme systems using more specific inhibitors. In the early quantitative studies of the effect of cyanide on oxygen consumption it was noted that low concentrations stimulated respiration (Meier, 1927; Resnitschenko, 1927; Hyman, 1919; Kisch, 1933). Later investigations using both cyanide and carbon monoxide con-

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firmed these observations (Orström, 1935; Lindahl, 1939; Borei, 1939; Commoner, 1939; Stannard, 1940). The existence of a maximum in the rate-concentration curve was noted by Krahl and Clowes (1935) in the case of the effect of dichlorophenol on the fermentation rate of yeast. The data of Commoner and Thimann (1941) on the effect of auxin and iodoacetic acid on the respiration and growth of the *Avena* coleoptile show similar phenomena.

Although comparatively rare, the phenomenon has also been observed in experiments with isolated enzyme preparations. Glick and King (1932) and Sobotka and Glick (1934) reported that octyl alcohol increased the rate of tributyrin hydrolysis by liver esterase at one concentration and inhibited it at a higher concentration. Michaelis and Stern (1931) noted the same thing in their study of the influence of iron salts on katepsin.

The tendency to overlook this effect frequently produces needless controversy; experiments over one small range of concentrations by one author appear to contradict the results of another obtained over a different concentration range. Where these opposite effects have not been completely ignored, the disposition has been to explain their existence by assuming that the substance in question exerts its effects *via* different mechanisms at high and low concentrations. Thus Winzler (1944), in commenting on the stimulatory effect of certain concentrations of cyanide and carbon monoxide on respiration, states "... it is well to keep in mind that they (the respiratory poisons) may have other unrelated effects." It is undoubtedly true that the specificity of most reagents and in particular of even the so called "specific inhibitors" may now be seriously questioned. And, it is not unlikely that the same substance may exert its effect *via* different components of *e.g.* the respiratory mechanism. Nevertheless, the necessity for postulating entirely unrelated mechanisms for the opposing effects at different concentrations has clearly not been established. This could only be done by demonstrating that the acceptable kinetic schemes of metabolic activity do not contain extrema, and in particular maxima, in the substrate rate-concentration curves derivable from them. Thus far no such analyses have been offered.

In view of this situation and of the wide generality of the phenomenon, it seemed worthwhile to investigate the possibility that relatively simple physico-chemical mechanisms may be responsible for the rate-concentration maxima. For purposes of comparison, certain mechanisms are included which might seem to provide adequate conditions for the existence of the phenomenon, but which on closer analysis fail to do so.

It is important to emphasize that it is the purpose of the present paper, not to assert the relevance of a unique mechanism, but merely to present some which do parallel the phenomenon. Their exhibition then, opens up the possibility of explaining the phenomenon without invoking unknown and unrelated effects.

## EXPERIMENTAL

*A. Materials and Methods.*—

Regeneration rate of hydranths was measured by the ratio  $L/t$ , a method first proposed by Barth (1938). In this,  $L$  is the length of the regenerating primordium and  $t$  is the time in hours from the removal of the old hydranth to the appearance of a constriction between the primordium of the new hydranth and the rest of the stem.

The solutions used were made up fresh each week in filtered sea water, and when necessary were adjusted to pH 8.2 with HCl. Young, unbranched stems, uniform in translucence, length, and diameter were selected from colonies freshly gathered from the waters of Vineyard Sound or Cape Cod Bay during the months of July and August. Stem segments 6 mm. in length were cut from regions about 5 mm. proximal to the hydranth. Groups of 25 stem segments were kept in 100 ml. of the appropriate solutions in partly filled, tightly stoppered flasks which were shaken at intervals to redistribute the oxygen. Solutions were changed daily, but the stems were kept in the flasks until they reconstituted or were finally transferred to fresh sea water, after from 4 to 5 days. They were counted as totally inhibited, with regeneration rate zero, if after being transferred they developed hydranths.

*B. Results.*—Table I summarizes the data obtained with phenylurethane. To arrive at a clearer picture of the phenomenon, the data of Experiment 1 are plotted (Fig. 1) in terms of per cent of normal against the logarithms of the molar concentrations multiplied by  $10^6$ . It is seen from the table that a maximum occurs in the neighborhood of  $1 \times 10^{-3}$  molar. It was generally noted that although the height of the maximum with any given compound might vary considerably, the concentration range in which it occurred was relatively restricted and reproducible.

In an effort to further test the reproducibility of the phenomenon, similar experiments were performed with ethylurethane, a narcotic closely related to phenylurethane. Since its general mode of activity would presumably not be very different from that of phenylurethane, it was to be expected that ethylurethane would also yield maxima in the rate-concentration curve. Table II summarizes the results obtained over a concentration range comparable to that covered in the phenylurethane experiments. Here again maxima are exhibited and the concentration range in which they occur is relatively narrow. It is of some interest to note that the maxima occur at about the same molar concentration for both narcotics.

The data supply further evidence to the already impressive collection for the reality of the phenomenon. Its reproducibility in these measurements of a complex biological process encouraged the attempt at a search for possible mechanisms. While the data presented here supplied the stimulus for the theoretical investigations of the next section, the analysis is more general in scope. It has as its major purpose the discovery of relatively simple physico-chemical systems which would exhibit rate-concentration maxima. In the

process, it will be seen that structural restrictions are one of the peculiarities possessed by those systems which do exhibit these effects.

TABLE I  
*The Effect of Various Concentrations of Phenylurethane on Regeneration Rates*

Experiment No.	Concentration	No. of stems	L/t	Per cent of Control
1	<i>mols/liter</i>			
	Control	21	42.9	100
	$1 \times 10^{-7}$	22	42.0	98
	$1 \times 10^{-6}$	24	42.9	100
	$5 \times 10^{-6}$	19	42.0	98
	$1 \times 10^{-5}$	17	44.2	103
	$1 \times 10^{-4}$	20	53.0	120
	$1 \times 10^{-3}$	21	57.4	134
	$1 \times 10^{-2}$	18	48.0	112
	$2.5 \times 10^{-2}$	20	41.5	97
	$5 \times 10^{-2}$	22	33.4	78
	$8 \times 10^{-2}$	20	23.2	54
2	Control	24	31.6	100
	$1 \times 10^{-7}$	22	31.0	98
	$1 \times 10^{-6}$	23	32.0	101
	$1 \times 10^{-5}$	22	32.4	102
	$5 \times 10^{-5}$	20	37.0	117
	$1 \times 10^{-4}$	21	40.7	129
	$5 \times 10^{-4}$	19	45.9	145
	$1 \times 10^{-3}$	17	48.5	153
	$5 \times 10^{-3}$	19	42.1	130
	$1 \times 10^{-2}$	18	34.2	108
	$5 \times 10^{-2}$	14	22.1	70
	$8 \times 10^{-2}$	14	10.7	34

## THEORY

### 1. Negative Cases

*Case A.*—The substance whose concentration effect is to be studied (hereafter referred to as the critical substance) either can be used as a substrate or reacts to yield a substrate for the reaction whose rate is being examined. At the same time the critical substance forms a compound with the enzyme governing the principal reaction, thus inactivating it. Intuitively it might seem likely that increasing the critical substance would first accelerate the reaction by furnishing additional substrate, and then, as it inactivates more and more enzyme, eventually choke off the reaction at the higher concentrations.

We adopt the following notation for concentrations:  $c$  for the critical substance,  $s$  for the substrate which it produces,  $e$  the free enzyme,  $a$  the activated complex of enzyme and substrate,  $p$  the product, and  $a'$  the inactive complex of enzyme and critical substance,  $e_0$  being the total amount of enzyme present. The chemical equations then are:

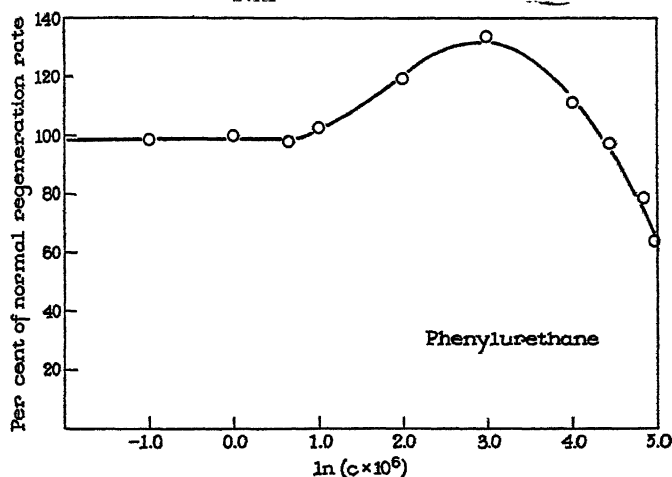
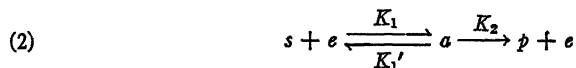
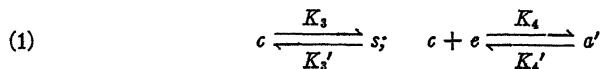


FIG. 1

In the above equations the  $K$ 's denote velocity constants of the corresponding reactions.

We now have the steady state<sup>1</sup> equations, which state for the various substances that the net reaction rate producing them equals the net reaction rate consuming them (if no diffusion out of the system takes place; when such dif-

<sup>1</sup> It is to be noted that throughout this paper transient (time-dependent) phenomena, in which a reaction begins at a finite level and eventually drops to zero, are not being considered. Stationary states only are compared, in each of which the concentration of the critical substance is different, but is and remains constant within any given state. It is very easy for confusion to arise between the two; and there are many phenomena, such as certain chains of radioactive transformations, which show maxima with respect to variations in time, but none in their steady state values.



TABLE II

*The Effect of Various Concentrations of Ethyl Urethane on Regeneration Rates*

Experiment No.	Concentration	No. of stems	<i>L/t</i>	Per cent of control
1	<i>mols/liter</i>			
	Control	25	24.7	100
	$1 \times 10^{-7}$	21	24.2	98
	$1 \times 10^{-6}$	20	24.7	100
	$1 \times 10^{-5}$	23	24.0	97
	$2 \times 10^{-5}$	18	24.2	98
	$5 \times 10^{-5}$	16	24.0	97
	$1 \times 10^{-4}$	19	25.1	101
	$4 \times 10^{-4}$	22	25.7	102
	$1 \times 10^{-3}$	18	29.0	118
	$5 \times 10^{-3}$	22	27.5	106
	$1 \times 10^{-2}$	21	22.2	90
	$5 \times 10^{-2}$	17	10.2	42
	$8 \times 10^{-2}$	14	4.9	20
2	Control	22	22.5	100
	$1 \times 10^{-7}$	18	22.0	98
	$1 \times 10^{-6}$	21	21.7	97
	$6 \times 10^{-6}$	17	22.2	99
	$1 \times 10^{-5}$	19	22.0	98
	$5 \times 10^{-5}$	20	22.5	100
	$1 \times 10^{-4}$	23	23.0	101
	$5 \times 10^{-4}$	18	21.7	97
	$1 \times 10^{-3}$	17	25.2	112
	$5 \times 10^{-3}$	20	22.0	98
	$1 \times 10^{-2}$	21	18.3	82
	$5 \times 10^{-2}$	17	8.3	37
3	Control	19	27.6	100
	$1 \times 10^{-4}$	21	28.7	102
	$5 \times 10^{-4}$	24	26.6	97
	$1 \times 10^{-3}$	19	32.0	116
	$5 \times 10^{-3}$	22	28.7	102
	$1 \times 10^{-2}$	21	25.1	91
	$5 \times 10^{-2}$	23	13.1	51

fusion occurs, the rate of inflow or outflow must be included in the material balance equations). These are:

$$(4) \quad K_3c - K_3's + K_1'a - K_{1se} = 0$$

$$(5) \quad K_4ce - K_4'a' = 0$$

$$(6) \quad K_{1se} - K_1'a - K_2a = 0$$

The critical substrate  $c$  is assumed to be maintained at a constant concentration in the system. Solving equations (3) to (6), we get:

$$(7) \quad a = \frac{\{(mc + n) \pm [(mc + n)^2 - 4K_2e_0K_2c]^{\frac{1}{2}}\}}{2K_2}$$

$$(8) \quad m = K_3 + \frac{(K_1' + K_2)K_2'K_4}{K_1K_4'}$$

$$(9) \quad n = K_2e_0 + \frac{(K_1' + K_2)K_2'}{K_1}$$

$$(10) \quad e = \frac{K_4'(e_0 - a)}{K_4c + K_4'}$$

$$(11) \quad s = \frac{K_3c - K_2a}{K_2'}$$

Equation (7) presents two solutions (both positive and therefore admissible). The one corresponding to the positive square root increases indefinitely with  $c$ . The solution corresponding to the negative square root approaches, as  $c$  is increased without bound, the saturation value  $K_3e_0/m$ . The conclusion that no maximum with respect to  $c$  exists can easily be confirmed by taking the derivative of (7) and setting it equal to zero; since the principal reaction in which we are interested is the production of  $p$ , whose rate is  $K_2a$ , and therefore proportional to (7). Why the second case does not result in a final zero reaction rate may be seen by examining (10) and (11) (whose product gives the rate at which the activated complex  $a$  is formed). As  $c$  is increased,  $e$  approaches zero, but  $s$  becomes infinite correspondingly fast, so that the product approaches a constant value. If an enzyme molecule is momentarily freed, it is certain to be seized upon by one of the dense horde of substrate molecules present.

This is of course the case only if the inactivation of enzyme is reversible. If it is made irreversible by setting  $K_4'$  equal to zero, it is evident from (5) that  $e$  will be zero in the *steady state* for all finite values of  $c$  and  $s$ , and so will  $a$ ; this may be confirmed by inspecting (8), which becomes infinite, so that (7) is either infinite for all values of  $c$  (which is nonsense) or, if we take the negative square root, is identically zero for all values of  $c$ .

*Case B.*—In this case it is assumed that the critical substance is simply a substrate for the reaction being studied. Although it does not inactivate the enzyme, it might be supposed that with very high substrate concentrations the products of the reaction would pile up sufficiently for the back reactions to lower the rate. This case illustrates the necessity for a precise differentiation between transient maxima and those occurring in a time-independent state.

Using the same symbols as previously, the equations for this system take the form:



The steady state equations are:

$$(3) \quad K_1'a - K_1ce + K_2a - K_2'pe = 0$$

$$(4) \quad K_2a - K_2'pe = h(p - p_0)$$

$$(5) \quad e_0 = e + a$$

Here  $h$  is the permeability coefficient of the product,  $p$ , multiplied by the ratio of cell surface to cell volume, and  $p_0$  is the external concentration of product. Solving, we obtain:

$$(6) \quad p = \frac{\{[(K_1hc + r)^2 + 4K_2'h(sK_1c + t)]^{\frac{1}{2}} - (K_1hc + r)\}}{2K_2'h}$$

$$(7) \quad r = (K_1' + K_2)(K_2' + h) - K_2'(K_2e_0 + hp_0)$$

$$(8) \quad s = K_2e_0 + hp_0$$

$$(9) \quad t = hp_0(K_1 + K_2)$$

$$(10) \quad e = \frac{s - hp}{K_2 + K_2'p}$$

The rate of production of  $p$  from  $a$  is the principal reaction by (4). It is equal to  $h(p - p_0)$ , and so will have a maximum if  $p$  has a maximum. But it is evident from (6) that, as  $c$  is increased,  $p$  will approach a saturation value. The other solution, with a negative square root, has been discarded because it leads always to negative and therefore meaningless values of the concentration  $p$ . If one had expected that increasing  $c$  would first act to increase the formation of  $a$ , and then, by increasing  $p$ , raise the back reaction in equation (2) to the point of halting the reaction, it is evident that this will not be the case. Whatever the permeability of  $p$ , it will always diffuse out in sufficient quantities so that, if a steady state is attained at all, it will exhibit a non-zero reaction rate no matter how large  $c$  is made. However, it may be noted that transient maxima in the rates may occur during the time in which the system is approaching the steady state. It is evident from (4) that, if there is no diffusion whatever, the reaction will in the steady state be in true equilibrium with a zero reaction rate; but this will be the case for any value of  $c$ . The total amount of  $p$  present in this equilibrium state will be simply proportional to  $c$ .

*Case C.*—For future reference, as well as comparison with case A, it is of interest to examine the following for maxima:  $s$ , the substrate, is adsorbed on a surface, where it reacts to form a product  $p$ , which is then desorbed. The "poison" or critical substance stimulates the production of  $s$ , but also inactivates the enzyme by reversible adsorption on its surface. While this system is formally analogous to that of case A, emphasis is here placed on the kinetics at the activating surface in terms of active centers and thus permits the analysis and detection of any differences which may stem from the heterogeneity of the system. The steady state equations are:

$$(1) \quad g' N_e = gc (N - N_s - N_e)$$

$$(2) \quad VKc = as (N - N_s - N_e) - a' N_s$$

$$(3) \quad LN_s = as (N - N_s - N_e) - a' N_s$$

$$(4) \quad Vc^* = V_e + N_e$$

$N$  is the number of free places on the surface.  $g$  is the adsorption coefficient and  $g'$  is the desorption coefficient of  $c$ .  $c$  and  $s$  refer to concentrations of critical substance and substrate in solution, while  $N_e$  and  $N_s$  refer to the corresponding number of molecules adsorbed on the surface.  $c^*$  is the total concentration of "poison."  $L$  is the reaction constant of  $s$  on the surface. The solution of the system is given by:

$$(5) \quad c = \frac{\{(fc^* - m) - [(fc^* - m)^2 + 2f'c^*n]^{\frac{1}{2}}\}}{n}$$

$$(6) \quad n = 2f \left( 1 - \frac{K}{L} \right)$$

$$(7) \quad m = gN + f'$$

$$(8) \quad f = gV; \quad f' = g'V$$

$$(9) \quad LN_s = VKc$$

It is evident here, as in case A, that the reaction rate, which is proportional to (5) by (9), either becomes infinite with the critical concentration  $c^*$ , or attains a saturation value, in no case giving a maximum.

*Case D.*—Again for purposes of later comparison as well as a further investigation into the consequences of heterogeneity, consider the following case: a substrate  $A$  is adsorbed on an enzyme surface; another substance  $B$ , if it colloides with an activated  $A$  molecule on the surface, reacts with it to form a product  $P$ , which is then desorbed. Here either  $A$  or  $B$  may be considered as the experimentally varied critical substance. With the notations  $c_a$  and  $c_b$  for concentrations in solution,  $N'$  for number of  $A$  molecules adsorbed on the surface, and  $N_p$  for number of product molecules on the surface, and  $N$  for total number of places on the surface, we have the steady state equations for

the number of free places on the surface and for the number of places occupied by product molecules:

$$(1) \quad ac_a (N - N_a - N_P) - a' N_a - KN_P = 0$$

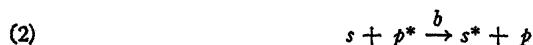
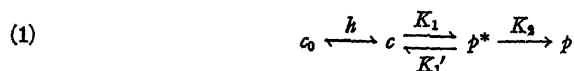
$$(2) \quad bC_b N_a - KN_P = 0$$

The solution is:

$$(3) \quad n_p = \frac{abc_a c_b N}{[K(ac_a + a') + bc_b(ac_a + K)]}$$

The rate at which product  $P$  is released into solution, which is proportional to (3), behaves the same way with respect to  $c_a$  and  $c_b$ . As either one is increased, the rate approaches a saturation value, and exhibits no maximum.

*Case E.*—An extremely interesting system to analyze, and one of some physiological importance, is one in which the critical substance  $c$  is involved in a reaction which is coupled to the over-all one whose rate is being studied; *i.e.*, supplies an activated complex utilized for substrate transformation in the main reaction. The critical substance  $c$  diffuses in and an excited or activated complex  $p^*$  is formed (Equation 1, below). This is then deactivated by collision with either the solvent, or with the reactant or product of the main reaction (Equations 2 and 3 below). The substrate  $s$  of the main reaction is kept at a constant concentration, and goes over into a product  $u$ , which is removed at a rate proportional to the difference between the internal and external concentrations ( $u - u_0$ ) (Equation 4). The schematic diagram for this system with the appropriate velocity constants and employing the subscript 0 for external concentrations may be written:



The steady state equations for the critical substance and the activated complex take the form:

$$(5) \quad h(c_0 - c) = K_1 c - K_1' p^* = K_2 p^*$$

where

$$(6) \quad K_2 = a + bs + gu$$

In (6)  $a$  is the deactivation coefficient for collisions of  $p^*$  with solvent molecules, whereas  $b$  and  $g$  perform the same function for collisions with  $s$  and  $u$  respectively (see (2) and (3)). The steady state equation for the substrate transformation takes the form:

$$(8) \quad k_1 s - k_1' u = m(u - u_0)$$

For purposes of calculation  $k_1$  and  $k_1'$  may be put in the form

$$(8) \quad k_1 = a_1 + b p^*; \quad k_1' = a_1' + g p^*$$

The  $a_1$  in the above measures the contribution to activated substrate,  $s^*$ , due to collisions with solvent and this is added to those obtained by collision  $p^*$ . Similarly  $a_1'$  measures the amount of back transformation of  $u$  into  $s^*$  through collisions with solvent in addition to that which occurs because of collisions of  $u$  with  $p^*$ . Since the over-all rate, according to equation (7), may be put in the form  $m(u - u_0)$ , we may solve for  $u$  as a function of  $c_0$  and examine for maxima. Solving, we get  $u$  of the form:

$$(9) \quad u = (Ac_0 + B) + [(Ac_0 + B)^2 + (rc_0 + D)]^{\frac{1}{2}}$$

where  $A$ ,  $B$ ,  $r$ , and  $D$  are constants which are combinations of the other parameters.

Differentiating  $u$  with respect to  $c_0$  in the above it is clear that no maximum exists and the curve is of the saturation type. If we suppose that  $c$  is identical with  $u$ , we obtain all the equations the same as before (with  $c$  instead of  $u$ ), except the very first, which becomes:

$$(10) \quad k(u_0 - u) = K_1 u - K_1' p^* + k_1' u - k_1 s$$

Equation (7) disappears. The result, however, is the same as above, with no maximum.

It is important to realize for later reference that homogeneity has been assumed throughout the analysis of the present case. All activations and deactivations were assumed effected by collisions between molecules in a homogeneous system.

## 2. Positive Cases

The following cases do exhibit maxima in the rate-concentration curve. It will be noted that they are modifications of those already discussed in the previous section. The detailed comparison will be considered in the discussion.

*Case A'.*—Let us suppose that two substrates,  $A$  and  $B$ , either of which may be considered as the critical substance, are present in solution in concentrations  $c_a$  and  $c_b$ . A single large enzyme molecule of surface area  $S$  is present, containing  $N$  identical sites, upon which substrate is adsorbed; the number of

adsorbed molecules of each species shall be denoted by  $N_a$  and  $N_b$ . In order that  $A$  and  $B$  may react, they must be upon neighboring sites of the surface. The product molecules are desorbed at a certain rate.

Two cases are possible. The first which we treat is one in which adsorption equilibrium is rapidly attained in comparison with chemical reaction, so that we may calculate this equilibrium without taking the reaction rate into account.

If the coordination number (number of sites surrounding a given site) of the surface is  $z$ , the average number of neighboring pairs of sites on the surface is:

$$(1) \quad n_{ab} = \frac{1}{2} zN$$

The average number of neighbor sites occupied at any time by an  $A$  and a  $B$  molecule is given by this, multiplied by the probability that a neighbor pair is so occupied. This is given by:

$$(2) \quad P_{ab} = P(a1)P_{a1}(b2) + P(b1)P_{b1}(a2)$$

In this notation,  $P(a1)$  is the probability of finding an  $A$  molecule on site 1 of a neighbor pair.  $P_{a1}(b2)$  is the conditional probability that a  $B$  molecule is on site 2 if an  $A$  molecule is on site 1. A similar interpretation is obvious for the other two symbols. If we assume for the sake of simplicity that the presence of an  $A$  or  $B$  molecule in a given region of the surface does not influence the adsorption of others, except insofar as it occupies a site, we obtain for the probabilities:

$$(3) \quad P(a1) = \frac{N_a}{N}; \quad P(b1) = \frac{N_b}{N}$$

$$(4) \quad P_{a1}(b2) = \frac{N_b}{N-1}; \quad P_{b1}(a2) = \frac{N_a}{N-1}$$

We thus obtain:

$$(5) \quad P_{ab} = \frac{2N_a N_b}{N(N-1)}$$

Thus we have for the average number of  $A$ - $B$  neighbor pairs on the surface:

$$(6) \quad N_{ab} = n_{ab} P_{ab} = \frac{N_a N_b z}{(N-1)}$$

The reaction rate on the surface will be denoted by:

$$(7) \quad R = KN_{ab}$$

We now write the steady state equations, which state that the rate at which  $A$  is adsorbed on the surface (proportional to the number of free places and to its concentration in solution) is equal to the rate of desorption, and similarly for  $B$ .

$$(8) \quad a c_a (N - N_a - N_b) = a' N_a$$

$$(9) \quad b c_b (N - N_a - N_b) = b' N_b$$

We solve this system, assuming  $c_a$  and  $c_b$  to be maintained constant, and get:

$$(10) \quad N_a = \frac{Na'b'c_a}{a'b'c_b + b'(ac_a + a')}$$

$$(11) \quad N_b = \frac{Na'b'c_b}{a'b'c_b + b'(ac_a + a')}$$

Combining the solutions with (7), we find for the reaction rate:

$$(12) \quad R = \frac{Mc_ac_b}{[a'b'c_b + b'(ac_a + a')]^2}$$

$$(13) \quad M = \frac{Kab'a'b'zN^2}{(N-1)}$$

$R$  is evidently the same considered as a function of either  $c_a$  or  $c_b$ . It begins at zero, rises, and drops off again to zero as the concentration increases indefinitely. This is confirmed by differentiation; choosing  $A$  as the critical substance, differentiating with respect to  $c_a$  and setting the result equal to zero, we obtain:

$$(14) \quad c_a = \frac{a'b'c_b + a'b'}{ab'}$$

The larger  $c_b$ , the larger is the value of  $c_a$  for which  $R$  attains a maximum and begins to decrease. The interpretation is quite clear: for some finite value of  $c_b$ , if we begin with no  $A$  present and gradually increase its concentration, the number of  $A$ - $B$  pairs formed on the surface will increase. Then, as  $c_a$  increases still further,  $A$  molecules will tend to occupy more and more of the surface, leaving less and less room for  $B$  molecules (as is clear from (11), since  $N_b$  tends to zero with increasing  $c_a$ ). Since it is necessary for  $A$  and  $B$  molecules to become neighbors on the surface in order that the reaction may proceed, this implies that the reaction rate will drop off for lack of  $A$ - $B$  pairs to work with, and eventually become zero.

*Case B'.*—In case  $A'$  we considered that the adsorption equilibrium on the enzyme surface was attained quite rapidly as compared with the reaction rate. We now modify this for cases where the rates are comparable. If a fraction  $m$  of the  $AB$  molecules produced per second is desorbed, the desorption rate is  $mkN_{ab}$ , which is calculated from equation (6) of case  $A$ . The steady state equations each assert that the rate at which substrate is adsorbed is balanced by the rate at which it is desorbed free plus the rate at which it is desorbed in the form of the  $AB$  compound:

$$(1) \quad Ac_a(N - N_a - N_b) = a'N_a + LN_aN_b$$

$$(2) \quad b'c_b(N - N_a - N_b) = b'N_b + LN_aN_b$$

$$(3) \quad L = \frac{mKz}{(N-1)}$$



This system may be solved, and  $N_{ab}$  calculated. We first introduce some abbreviations:

$$(4) \quad G = LNb'$$

$$(5) \quad f = a'(bc_b + b') + LNbc_b$$

$$(6) \quad f' = f - 2LNbc_b$$

Taking  $A$  as the critical substance, and using the notation  $x$  for  $ac_a$ , we have:<sup>2</sup>

$$(7) \quad 2LN_a = \frac{\{[f + (b' - LN)x]^2 + 4Gx(a' - bc_b + x)\}^{\frac{1}{2}} - f - (b' + LN)x}{(a' - bc_b + x)}$$

$$(8) \quad 2LN_b = \frac{\{[f + (b' - LN)x]^2 + 4Gx(a' - bc_b + x)\}^{\frac{1}{2}} - f' - (b' + LN)x}{(b' + bc_b - x)}$$

The reaction rate is proportional to the product of (7) and (8). To determine the presence of a maximum, one should differentiate the product and set the result equal to zero, solving for  $x$ . The result is an equation of unwieldily high degree; it appears preferable to study the rate by semiquantitative methods. We shall do this by analyzing (7) and (8) separately, whereupon it will be possible to draw certain conclusions regarding the behavior of the product.

Considering (7) plotted against  $x$ , it appears that  $N_a$  is zero when  $x$  equals zero, and approaches the limiting value  $N$  as  $x$  becomes infinite. In this range of  $x$ , the denominator of (7) may change sign if  $(a' - bc_b)$  is negative. But this does not affect the sign of  $N_a$ ; when the denominator is positive, the square root in the numerator is larger than the rest of the numerator, so that the quotient is positive. When the denominator is negative, the square root is less than the remainder of the numerator; numerator and denominator are both negative, and the fraction again positive. It remains only to determine whether (7) has a singularity for that value of  $x$  at which the denominator is equal to zero. The limit is easily found by the usual procedure of differentiating numerator and denominator separately, and turns out to have the finite value:

$$(9) \quad N_a' = \frac{Nb'(bc_b - a')}{[f + (b' - LN)(bc_b - a')]}$$

For this same value of  $x$ ,  $N_b$  also has a finite and positive value,  $Na'/(a' + b')$ . The slope of the  $N_a - x$  curve at the origin is equal to  $Nb'/^3a'(b' + bc_b) + LNbc_b^3$ , which is positive. The slope when  $x$  becomes very large goes to zero as  $1/x^2$ . It is evident from this that  $N_a$  rises from zero through positive values to the asymptotic value  $N$ .

<sup>2</sup> The solutions for  $N_a$  and  $N_b$  with the minus sign of the radical yields negative values for either  $N_a$  or  $N_b$  or both and are therefore not considered.

At  $x$  equals zero,  $N_b$  has the finite positive value  $Nbc_b/(bc_b + b')$ . For very large values of  $x$  it goes to zero as  $1/x$ . Investigating the zero of the denominator we find at this point the value:

$$(10) \quad N_b' = \frac{Na'bc_b}{[(a' + b')(b' + bc_b) + LNb']}$$

This is positive. For the same value of  $x$ ,  $N_a$  has the positive value  $Nb'/(a' + b')$ . The initial slope of the  $N_b - x$  curve is given by the initial slope of the  $N_a - x$  curve multiplied by the factor  $-bc_b^2(b' + bc_b)^{-1} + LN(b' + bc_b)^{-2}$ . The slope is therefore negative. For very large values of  $x$ , the slope goes to zero as  $-1/x^2$ . The  $N_b$  curve consequently begins at a finite value and drops off asymptotically to zero.

One function is finite and the other zero at the origin; their product is therefore zero at the origin. One function is zero and the other finite for infinite values of  $x$ ; their product is therefore zero for infinite values of  $x$ . They are positive and free of singularities between the extremes of the  $x$  range. Consequently, there must exist at least one maximum of their product for some positive and finite value of  $x$ . If either of the two factors is not monotonic, there may be more than one maximum.

*Case C'.*—It was pointed out in the analysis of case E that the assumptions which led to the steady state equations of the system were based purely on reactions in a homogeneous system. It is clearly of no little importance to examine the coupled reaction system modified to consider the effects of imposing geometrical constraints by assuming that the reactions take place on enzyme surface. Using the same reaction system we assume here that  $c$  is adsorbed on an enzyme as  $c_e$  and is activated as  $p^*$ . The latter goes into  $p_e$  which is then desorbed. The deactivation of  $p^*$  takes place by transfer of energy to  $s$ , which is adsorbed on the enzyme surface as  $s_e$ . The transfer of energy converts  $s_e$  to  $s^*$ , which then transforms to  $u_e$ , which is then desorbed as  $u$ . If  $N_e$  is the total number of places on the enzyme and  $N_0$  the number of free places, the assumption of time independence for adsorption and activation of  $c_e$  leads to

$$(1) \quad \alpha cN_0 = k_e c_e$$

where  $\alpha$  is the adsorption coefficient and  $k_e$  the activation coefficient. Setting activation and deactivation rates equal yields:

$$(2) \quad k_e c_e = Lp^* + k s_e p^*$$

where  $L$  is the deactivation coefficient when the energy is not transferred to the adsorbed substrate  $s_e$ , and  $k$  is the deactivation coefficient when  $p^*$  transfers its energy to the substrate. Setting the deactivation rate of  $p^*$  equal to the rate of desorption of the deactivated product  $p_e$  gives:

$$(3) \quad Lp^* + k s_e p^* = b p_e$$

where  $b$  is a desorption coefficient. Turning now to the substrate  $s$  of the main reaction, the net rate of adsorption is set equal to the rate of substrate activation; thus

$$(4) \quad g s N_0 - g' s_e = k_s p^*$$

where  $g$  and  $g'$  are the adsorption and desorption coefficients respectively, and  $k$  is the activation coefficient of the adsorbed substrate. Finally, setting the activation rate equal to rate of inactivation of the substrate and the desorption of the resulting product  $u_e$ , we obtain:

$$(5) \quad k s_e p^* = e s^* = d' u_e$$

The desired reaction rate  $R$  of the main reaction is given by  $d' u_e$ . The solutions for  $R$  as a function of  $c$ , the concentration of the critical substance, are given by;

$$(6) \quad R = -\frac{1}{2} \frac{B}{A} + \left[ \left( \frac{1}{2} \frac{B}{A} \right)^2 - \left( \frac{D}{A} \right) \right]^{\frac{1}{2}}$$

where

$$(7) \quad \begin{cases} A = (ac + b')(a'c + b) \\ B = \left( \frac{k_s N_0 g s}{\alpha} \right) (a'c + b) + k_s N_0 c (ac + b') + \left( g' \frac{L}{k} \right)^2 (ac + b)^2 \\ D = \left( \frac{g s}{\alpha} \right) (k_s N_0)^2 c \end{cases}$$

in which the constants  $a$ ,  $a'$ ,  $b$ , and  $b'$  are combinations of the other constants. It is seen that  $R$  is zero for zero and infinite values of  $c$ , and consequently an extremum of the rate concentration curve exists. Without going into the algebraic detail, it is sufficient here to note that under the restriction that  $R$  be real and positive, this extremum is a maximum if  $A$  and  $B$  are both negative or of opposite sign and is a minimum if they are both positive.

*Case D'.*—Of some interest, in view of possible relations between enzyme activity and protein denaturation, is the case where the critical substance can not only inactivate the enzyme, but also may protect it from denaturation. Thus, suppose an enzyme is present in total concentration  $E_0$ . The reaction being studied takes place at a rate  $R$  proportional to the active concentration of enzyme,  $E_A$

$$(1) \quad R = k E_A$$

The enzyme is in equilibrium with its denatured and inactive form  $E_d$  described by

$$(2) \quad E_d = K_2 E$$

where  $E$  is concentration of free enzyme. A substance  $S$  also reacts with the enzyme. If it forms a compound  $E_s$  given by

$$(3) \quad E_s = K_1 S E$$

it protects the enzyme from denaturation. But if it reacts further with  $E_s$  to form  $E_{ss}$ , where

$$(4) \quad E_{ss} = K_2 S E_s$$

it occupies an essential group in the enzyme, and renders it inactive. The total concentration of enzyme  $E_0$  is given by:

$$(5) \quad E_0 = E + E_d + E_s + E_{ss}$$

and the active form  $E_A$  by

$$(6) \quad E_A = E + E_s$$

Solving for  $R$  as a function of  $S$ , the concentration of critical substance, and the velocity constants, we obtain,

$$(7) \quad R = \frac{k E_0 (1 + K_1 S)}{1 + K_2 + K_1 S + K_1 K_2 S^2}$$

Differentiating  $R$  with respect to  $S$  we find that  $R$  does have a maximum, which is attained when  $S$  has the value:

$$(8) \quad S_{\max.} = \frac{-1 + \left(1 + \frac{K_1 K_2}{K_2}\right)^{\frac{1}{2}}}{K_1}$$

which it is seen is greater than zero.

It will be noted that  $S_{\max.}$  increases with  $K_2$ , the denaturation constant of the enzyme. Thus, if heat denaturation is involved, one would expect a series of rate-concentration curves at different temperatures to have the location of their maxima displaced towards higher concentrations with increasing temperature.

This case is of interest as illustrating what may occur if an inhibitor acts on an enzyme both competitively and non-competitively with respect to the substrate. Even if the non-competitive poison partially inactivates the enzyme, a stimulating effect may be found. For if only a fraction  $f$  of  $E_s$  remains active, we get instead of (8):

$$(9) \quad S_{\max.} = \frac{-1 + \left[1 + \frac{f K_1}{K_2} \{1 - (1 + K_2) f\}\right]^{\frac{1}{2}}}{f K_1}$$

$S_{\max.}$  increases towards the value (8) as  $f$  approaches unity. It is equal to zero when

$$(10) \quad f = \frac{1}{1 + K_2}$$

For larger values of  $f$ , a maximum always occurs. The critical value of  $f$  is smaller the larger is  $K_3$ ; *i.e.*, we might say that a non-competitive poison will stimulate at low concentrations if its inhibitory effect on the enzyme is less severe than the effect of denaturation.

#### DISCUSSION

It is clear from the discussion of both the positive and negative cases that it is intuitively difficult to decide whether a particular mechanism will yield a maximum in the rate-concentration curve. A decision requires a detailed analysis of the steady state equations in all but the most naive situations.

An interesting characteristic which seems to emerge from the positive mechanisms analyzed here is the existence of a more definite type of geometrical constraint than was evident in the corresponding negative cases. Thus, if the positive cases  $A'$  and  $B'$  are compared with the parallel negative one  $D$ , it is seen that the geometrical conditions imposed on the reactions in the former two systems are more severe than those assumed in the case of  $D$ . The distinguishing mark of  $A'$  and  $B'$  is the proviso that the various substrate molecules must occupy certain relative positions on a surface in order to react at all. Again the homogeneous coupled reaction of case  $E$  which was negative was made positive (case  $C'$ ) by introducing the geometrical condition of heterogeneity. It is to be by no means concluded that the geometrical conditions of structure are *necessary* for the exhibition of maxima in rate concentration curves, although they are, as we have seen, sufficient if of a relatively rigid nature. Nevertheless, in view of the present study, it is perhaps not surprising to find such phenomena relatively common in the structured systems cells are presumed to be, whereas they are relatively rare in "test-tube" experiments. In any case, it is evident that the existence of a maximum in the rate-concentration curve of a substance does not necessarily imply a dichotomy in the mechanism of its activity at different concentration.

Interpretation of the experiments reported here cannot be made directly in terms of the positive cases described since it is hardly likely that the narcotics enter directly as substrates in the synthetic reactions of regeneration. However, the existence of maxima in their rate-concentration curves would be explained if they affected the enzymes involved in the formation of the substrates used in regenerative activity. The narcotics would thus influence the concentration of the critical substance whose variation leads to maxima.

One other important point may be noted; it is evident from the analysis that a unique mechanism for these maxima does not obtain. Hence, its existence in any particular instance is not diagnostic of a particular reaction mechanism. While certain types of reactions are ruled out for any process or system which exhibits a maximum, there remain too many positive possibilities for it to be particularly useful as a tool for investigating mechanisms.

## SUMMARY

1. Reproducible maxima are exhibited in the rate-concentration curves obtained by studying the effects of ethyl- and phenylurethanes on regeneration rates of hydranths in *Tubularia*.

2. The general problem of maxima in rate-concentration curves is analyzed in terms of reaction kinetics of relatively simple systems.

3. Certain systems were shown to exhibit this phenomenon. A comparison is made of these with similar ones which fail to do so.

4. The possible rôle in this phenomenon of cellular structure and its attendant geometrical constraints is discussed in terms of the above comparison.

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# THE EFFECT OF PENICILLIN ON EGGS OF THE SEA URCHIN, *ARBACIA PUNCTULATA*\*

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It has been shown (1, 2) that the general cell inhibitors urethane, chloral hydrate, and sulfanilamide inhibit cell division of fertilized *Arbacia* eggs, and that this inhibition results from an inhibition of the specific fraction of the cell respiration upon which cell division depends. It was considered to be of interest to study the effects of the chemotherapeutic agent, penicillin, on these cells.

## *Materials and Methods*

Eggs and sperm of the sea urchin, *Arbacia punctulata*, were obtained essentially as described by Just (3). Handling of the eggs and data, and the respiratory experiments were carried out as in previous reports (1, 2). The penicillin<sup>1</sup> solutions and the sea water used in the sea urchin egg experiments were adjusted to pH 7.5. This pH was selected because of the instability of penicillin at pH greater than 7.9 (4) (sea water has a pH of about 8.2), and because this increase in hydrogen ion concentration does not materially affect cell division of fertilized sea urchin eggs. The per cent of cells in a suspension was determined by high speed centrifugation of a 1 ml. sample in a hematocrit tube.

## *Effect on Cell Division*

Penicillin (P) inhibits cell division of the fertilized sea urchin egg. This inhibition is directly related to P concentration (Fig. 1), the inhibition at 250 units (U)/ml. being slight, inhibition at 3000 U/ml. being complete (for reasons explained later, the dotted line in Fig. 1 represents approximately the corrected relation between cell division and penicillin concentration in that range of concentrations). In this respect, the action of P on fertilized sea urchin eggs is identical with that of numerous other cell inhibitors such as urethane, chloral hydrate, and sulfanilamide. One very important difference between the action of P and these other inhibitors on fertilized sea urchin eggs is its much greater tendency to kill the cell (vacuolated cytoplasm, pigment clumps). As the con-

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<sup>1</sup> The penicillin used was the therapeutic preparation manufactured by Chas. Pfizer & Co., Inc. and put up in 100,000 unit sterile lots.



centration of P is increased in the range employed, more and more cells are found dead for any particular period of contact with the drug. Similarly with any one concentration of P, the number of cells killed increases with time. This is in distinct contrast to the other inhibitors mentioned, *e.g.* concentrations of sulfanilamide which completely inhibit division will kill very few cells even after several hours contact.

Tests were made of the reversibility of this inhibition. At a concentration of P of 7500 U/ml. reversal could be obtained by washing the cells in sea water after one-half hour's contact with the drug, but not after 1 hour's contact. This is not in reference to those cells which are obviously dead (vacuolated cytoplasm, pigment clumps, cytolysis, etc.), but in reference to cells which as

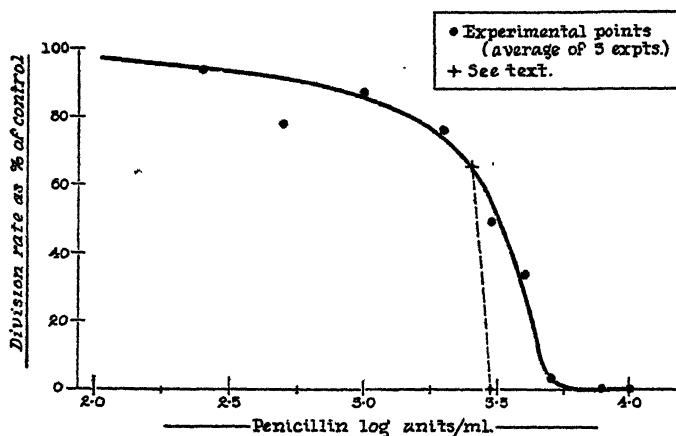


FIG. 1. Inhibition of cell division by penicillin. About 1 per cent cell suspension by volume.

far as appearance is concerned are normal. This also is in distinct contrast to the other inhibitors, *e.g.* an exposure longer than 70 minutes to a 0.34 M (3,029 mg. per cent) solution of urethane is required to cause irreversible damage, this concentration being almost three times that necessary to inhibit division completely (1). With 0.04 M (690 mg. per cent) sulfanilamide, which just completely inhibits division, complete reversal can be obtained even after 3 hours' contact (2).

It has been repeatedly stated (5-7) that P acts as a bacteriostatic on bacteria, assuming that it retards the rate of division. Actually, however, it is extremely difficult with cells which separate after division, to establish whether the decreased number of cells resulting from the action of inhibitors is due to a decreased rate of division of each cell or whether it is due to the killing or complete inhibition of some cells with others unaffected. With fertilized sea urchin eggs, however, the divided cells are still an intact entity and, therefore, it is

easy to determine in this case at least which of the two possibilities obtains. As seen in Fig. 2 penicillin decreases the division rate of each cell.

### *Penicillin on Oxygen Consumption*

Penicillin in concentration sufficiently high to inhibit cell division completely has no effect on the oxygen consumption of either unfertilized or fertilized eggs (11 determinations). Failure to inhibit oxygen consumption of bacteria has also been reported (8).

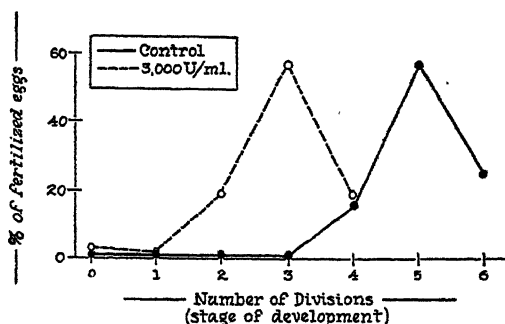


FIG. 2. Distribution of development of control fertilized eggs as compared with inhibited fertilized eggs. Average of three 3 hour experiments. 1 per cent egg suspensions.

### *Effect of Varying the Number of Cells per Unit Volume*

As seen in Fig. 3 there is an inverse relation between percentage of cell suspension and inhibition of division. A concentration of penicillin of 3000 U/ml. was chosen to test this relationship because in this immediate region any particular change in P concentration will result in the largest change in inhibition by P; *i.e.*, it is the region most sensitive to changes in P concentration (see Fig. 1). As seen in Fig. 3 there is considerable spread of points taken from different experiments. This would be expected for the same reason that this concentration was selected—its great sensitiveness.

On the assumption that the P concentration did not change upon the addition of the cells, and assuming that P inhibition is a result of adsorption of P onto some cellular component such as an enzyme, this inverse relationship between percentage of cell suspension and inhibition of division should not have been found. This statement deserves clarification. Let us place a single cell in a solution of P. Presumably the P is free to diffuse through the cell wall and into the protoplasm and there may adsorb onto one or more cell components; if this is the mode of inhibition by P, then the amount adsorbed is directly related to the inhibition produced. The cell wall, however, prevents the outward diffusion of the cell components. According to the principle of

mass action, the amount of P adsorbed onto or combined with one molecule of any one component (whether inside of or outside of a cell) is directly related to the concentration of P in solution and inversely related to the concentration of the component. Adding one more or a thousand more cells to this suspension of one cell we already have in no way alters the concentration of the component in each of the individual cells; the cells are separate entities. As already stated, it is assumed that the concentration of this component in the solution of P surrounding the cells is zero. Thus, as long as the concentration of P is kept constant (and not decreased appreciably by significant quantities of P being adsorbed from solution by the component in question), the inhibition produced must be independent of the number of cells in the suspension. The inverse relation is apparent, however, for which there are at least two possible explanations:

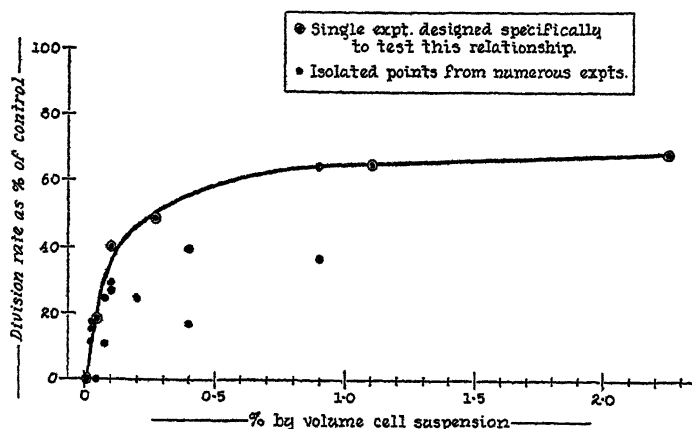


FIG. 3. Effect of percentage cell suspension on inhibition of division by 3000 U/ml. penicillin.\*

1. The cells are capable of destroying P, thus decreasing its concentration. This is made unlikely by the nature of Fig. 3; the curve should not approach a maximum.

2. Some cellular component (egg jelly? protein? enzyme?) is adsorbing (or dissolving) sufficient P from solution to decrease P concentration and thus cause the effect seen.

The second suggestion can be checked as follows; there are two possibilities:

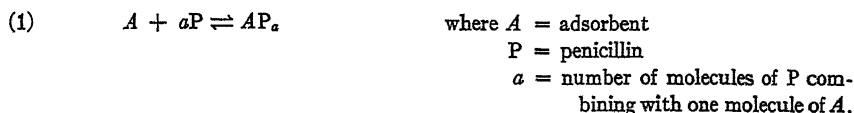
(a) The relation might depend upon solubility coefficients. This is not compatible, however, with Fig. 3. If P is more soluble in the egg than in the medium, then the maximum reached in the graph should not be. Conversely,

\* It is unfortunate that only one complete experiment designed to test this relationship was made. The isolated points from other experiments, however, definitely follow the same trend.

if the P is less soluble in the egg, then practically no P (less than 1 per cent) would be adsorbed by a 1 per cent suspension.

(b) The P is being adsorbed by some cellular component. As already explained, increasing the percentage of cell suspension increases proportionately the amount of adsorbing surface ( $A$ ) with which the P in solution comes into contact, but the concentration of  $A$  within each individual cell is not increased; this is a heterogeneous system.

When a cell suspension comes into contact with a solution of P,  $AP_a$  is formed until equilibrium is established according to Equations 1 and 2.



According to the law of mass action,

$$(2) \quad \frac{(A)(P)^a}{(AP_a)} = K.$$

The initial concentrations of  $A$  and P are thus decreased by amounts relatively equal to the amount of  $AP_a$  formed. If more cells are added, the  $A$  in these cells is all in the free, uncombined state. If

$$(3) \quad \frac{(A)(P)^a}{(AP_a)} > K$$

pertains, more  $AP_a$  is formed until the system again reaches equilibrium. Every time this happens the concentration of P is decreased, and as (P) decreases the tendency for P to adsorb onto  $A$  decreases because its pressure (concentration) is decreasing. Finally, P reaches a concentration below which it will not combine significantly with  $A$ , and once this point is reached, increasing the percentage cell suspension *ad infinitum* leads to no further decrease in the concentration of P. This is the condition seen in Fig. 3.

It is to be noted from Fig. 3 that as percentage suspension approaches 0 (as (P) approaches 3000), cell division rate approaches 0, thus cell division is completely inhibited at 3000 P (marked as + in Fig. 1). At suspensions greater than 1 per cent the true P concentration at 3000 U/ml. is at the critical concentration of P below which no P is bound by the cells (see Fig. 3). The percentage of control division rate at this critical concentration is about 65 per cent. From Fig. 1 it is seen that the concentration of P causing this amount of inhibition in a 1 per cent suspension is at log 3.42 P (about 2650 U/ml.); (this is both true and apparent concentration of P at this point). Points on the curve of Fig. 1 below this concentration are therefore true points. Points above this concentration are false because the concentration of P is

less than the critical concentration. The true curve in this region must approximate the dotted line.

Experiments (four in number) were set up as follows to determine experimentally whether P was being bound by the cells. The plan was to place an egg suspension in a concentration of P of 3000 U/ml. and then remove these eggs and determine the P concentration by growth assay. A 1.75 per cent suspension of fertilized eggs was permitted contact with a P concentration of 3000 U/ml. (solution 1) for one-half hour, at the end of which time the cells were removed by centrifugation. Now a 0.075 per cent cell suspension of fertilized eggs was allowed contact with the P solution to be assayed (solution 1), the inhibition to be compared with that produced on a similar suspension by a concentration of P of 3000 U/ml. (solution 2). A typical result is seen in Table I. This indicates definitely that the cells are binding P.<sup>2</sup> One other frequent observation which was made agrees with this interpretation. Growth experiments were run in Syracuse dishes with slightly concave bottoms. In the more concave dishes the cells would tend to aggregate near the center.

TABLE I

	Cell division rate as per cent of control
Solution 1 (unknown) .....	38
Solution 2 (3000 U/ml.) .....	11

In such instances the cells near the center were invariably inhibited to a considerably less degree than those scattered towards the periphery. The latter ones were usually all dead.

An important question is whether the combination of P with the cell which apparently accounts for the phenomenon just discussed is the actual point of attack directly responsible for the inhibition of cell division produced by P. Because of the fact that this adsorption takes place in the range of concentration where the inhibition curve is steepest (Fig. 1) this might at first seem likely. Results shown in Fig. 3, however, are incompatible with this interpretation. At cell suspensions greater than 0.9 per cent little or no more P is removed from solution by the cells beyond a maximum amount when an initial P concentration of 3000 U/ml. is used. Yet, it must be that as more cells are added the amount of P bound per cell decreases proportionately, since it is difficult to conceive in a presumably homogeneous cell suspension that some

<sup>2</sup> A 1 per cent suspension of *Arbacia* eggs has about 47,500 eggs/ml. (9). The density of these eggs is about 1.09; each milliliter of suspension has about 11 mg. of eggs. The manufacturer's assay of the penicillin sodium used in these experiments was approximately 755 U/mg. A concentration of 3000 U/ml. is, therefore, equivalent to 3.97 mg./ml. The standard of penicillin sodium, however, is 1650 U/mg.

cells bind P while others do not. The amount bound per cell in a 2 per cent cell suspension would be approximately one-half that bound per cell in a 1 per cent cell suspension. If the amount bound per cell decreases then the inhibition should decrease. The curve in Fig. 3, however, is asymptotic to a level of division rate inhibition of approximately 25 per cent. Another point against such an interpretation is seen in Fig. 1. It was calculated that at concentrations of P below approximately 2650 U/ml. little or no binding of P occurred, yet it is seen that concentrations of P as low as 250 U/ml. do inhibit cell division.

Hence, it is concluded that P is bound by the fertilized egg at least at two loci. One of these at concentrations of about 2650 U/ml. P and above, binds P in sufficient quantities to reduce significantly the concentration of P in solution and thus to account for the observed inverse relation between inhibition and the number of cells per unit volume; this binding is dissociated from the inhibitory effect of P. Undoubtedly then, the inhibitory effect results from a binding or adsorption of P on some other cellular component(s) but in this case the amount of P bound is so small that the P concentration is practically unchanged.

#### *Antagonism by Peptone*

Because it had been demonstrated that P could be bound in significant quantities by some cellular component, and because peptone antagonizes sulfonamide action in bacteria, it was decided to see whether P activity would be changed by peptone. It has been reported that it has no effect on P activity on bacteria (8, 10, 11).

There were no consistent indications that 1 per cent peptone antagonized P action (concentration 3000 U/ml.) but occasionally it had a slight antagonistic action, evidenced principally by the fact that in its presence fewer cells were killed. The peptone itself inhibited division slightly. 1 per cent egg albumin and 5 per cent sea urchin egg cytolysate were both inhibitory *per se* and synergized with P inhibition.

#### DISCUSSION

It seems fairly certain that penicillin achieves its inhibitory action on fertilized sea urchin eggs by a different mechanism than the inhibitors sulfanilamide, urethane, and chloral hydrate, previously studied. Cell division undoubtedly results from a chain of events, any one of which if blocked will interfere with division. One of these is the respiratory processes which provide the energy for the process, and apparently sulfanilamide, urethane, and chloral hydrate attack at this point. While it is conceivable that penicillin might inhibit this fraction of the total respiration and cause simultaneously an equivalent increase in the other respiratory fraction which is unconcerned with

division so that the over-all oxygen consumption is unchanged, it is probably unlikely. Penicillin then must attack some other vital link in the process of division. From these experiments it is impossible to determine the mechanism of this inhibition. The fact that it has a great tendency to kill the cell is probably of some significance. Charcoal experiments (12) indicate that penicillin is a surface-active substance.

In view of the facts that penicillin is surface-active, it is bound in relatively large amounts by some component of the sea urchin egg, and peptone has a slight antagonistic action in these experiments, it is quite probable that penicillin antagonists will be found in bacterial experiments which act by binding penicillin. In fact, there is evidence that penicillin is bound by some component of whole blood. In the experiments on decreasing penicillin excretion in the dog by simultaneous administration of *p*-amino hippurate (13), in some instances the renal clearance was less than the glomerular filtration rate.

The question can be raised regarding the high concentrations of penicillin required for inhibition of cell division as compared to those required for a similar effect on bacteria. Actually penicillin is a powerful inhibitor for the sea urchin egg. Penicillin sodium of a potency equivalent to the standard of 1650 U/mg. in a concentration of 180 mg. per cent would inhibit completely division of the sea urchin egg, whereas a concentration of 690 mg. per cent of sulfanilamide is required. Thus penicillin is four times as powerful an inhibitor for the sea urchin egg on a weight basis as sulfanilamide. Whether or not penicillin inhibits the sea urchin egg division by the same mechanism as it inhibits bacterial division is a question which cannot be definitely answered. The difference in concentrations required for inhibition in the two cases cannot be used as a criterion. From the observations made there is nothing to suggest that the mechanisms in the two cases differ.

#### SUMMARY

1. Penicillin in the range of concentration from 250 U/ml. to approximately 2650 U/ml. inhibits the rate of cell division of the fertilized sea urchin egg from 0 to 100 per cent.
2. Penicillin in the same range of concentrations has no effect on the oxygen consumption of the unfertilized or the fertilized eggs.
3. Penicillin is bound by some component of the sea urchin egg in amounts sufficiently large to lower the initial concentration, this binding apparently not being related to the inhibitory action.

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# THE EFFECT OF PENICILLIN ON METHYLENE BLUE ADSORPTION ONTO ACTIVATED CHARCOAL\*

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(Received for publication, January 31, 1945)

Sulfonamides are adsorbed onto charcoal (1, 2) and can inhibit the adsorption of methylene blue (MB) onto charcoal. Accordingly it was considered of interest to find out whether penicillin (P) would adsorb onto charcoal. Because of the difficulties in assaying for P the inhibition of MB adsorption was used as an indicator.

## *Materials and Methods*

The charcoal adsorption experiments were carried out as follows. Norit A was activated by heating at 100°C. for 24 hours after which it was kept in a desiccator with sodium hydroxide to remove CO<sub>2</sub> and calcium chloride to remove the moisture. Into 10 ml. volumetric flasks was placed first 0.7 ml. of a 1 per cent solution of methylene blue, followed by the penicillin, then distilled water added to the 10 ml. mark. The flasks were placed in a constant temperature water bath at 25°C. for 10 minutes, then removed and 0.100 gm. of charcoal added. The flasks were inverted three times and then placed in the bath again for exactly 10 minutes, at the end of which time the contents were filtered. The filtrate concentration of methylene blue was determined in a Klett biocolorimeter by comparison with known standards. With this technique the control filtrate usually contained about  $3 \times 10^{-5}$  per cent methylene blue. In the experiments to study the effect of peptone on P inhibition of MB adsorption onto charcoal, the same method was used. The peptone was added to the MB or to the MB plus penicillin in the flask before dilution to the 10 ml. mark with distilled water. A control without P or peptone was run for each experiment.

Control (without penicillin or peptone) = concentration of MB present in the filtrate  
in excess of saturation of the charcoal

## EXPERIMENTAL

P in concentration as low as 100 units/ml. inhibited MB adsorption onto activated charcoal. Table I shows data from four representative experiments, giving the concentration of MB in the filtrate with varying amounts of penicillin. The last column gives the figures showing how many times greater the concentration of MB in the filtrate was in the presence than in the absence of

\*This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

P. These values are used in showing graphically (Fig. 1) the relationship between P concentration and its inhibitory effect.

TABLE I

*Data from Four Typical Experiments Giving the Concentration of MB in the Filtrate in the Presence and Absence of P\**

Experiment No. and its symbol in Fig. 1	Concentration P	Per cent concentration MB $\times 10^4$	Concentration MB in presence of P
			Concentration MB in absence of P
1 (●)	<i>units/ml.</i>		
	0	0.32	—
	100	0.95	3.0
	200	2.15	6.7
	300	3.85	12.0
2 (+)	400	4.05	12.7
	0	0.50	—
	100	0.93	1.9
	400	4.62	9.3
	800	3.33	6.7
3 (Δ)	1000	4.40	8.8
	0	1.18	—
	100	2.12	1.8
	200	4.00	3.5
	400	5.98	5.1
4 (○)	800	10.2	8.7
	1000	10.6	9.0
	0	0.30	—
	100	3.00	10.0
	200	3.00	10.0
	300	2.40	8.0
	400	5.00	17.0
	600	7.20	24.0
	800	12.0	40.0
	1000	7.50	25.0

\* The experimental data are rather irregular. Experimental error is increased by the performance of experiments on a semimicro scale. This was necessary because of a limited supply of penicillin. Part of the variability probably was due to variation in the state of activation of the charcoal.

Since there had been a suggestion that peptone antagonizes P inhibition of fertilized sea urchin eggs (3), the effect of peptone on P inhibition of MB adsorption onto charcoal was studied. As seen in Table II, peptone in the concentrations used strongly inhibited MB adsorption *per se*, but P in much less inhibitory concentrations apparently antagonized the peptone inhibition. This

may be explained either by a specific surface phenomenon at the adsorbing charcoal surface or by an inactive complex formation between the P and the peptone.

TABLE II  
*Combination of Penicillin and Peptone on MB Adsorption by Charcoal*

In the presence of	(Per cent concentration of unadsorbed MB—per cent concentration unadsorbed MB in control*) $\times 10^4$
100 units/ml. P. . . . .	1
0.1 per cent peptone. . . . .	49
Both combined. . . . .	43
100 units/ml. P. . . . .	1
0.2 per cent peptone. . . . .	53
Both combined. . . . .	42
100 units/ml. P. . . . .	2.4
1 per cent peptone. . . . .	36
Both combined. . . . .	22
100 units/ml. P. . . . .	2.4
1 per cent peptone. . . . .	28
Both combined. . . . .	17

\* The control flask contained only charcoal and methylene blue (without either P or peptone).

#### DISCUSSION

As stated under Materials and methods, the MB concentration in each flask before adsorption took place was 0.07 per cent. It is apparent that the inhibition by P approaches a maximum and by examination of Table I it is found that the amount of MB whose adsorption is inhibited by the highest P concentrations relative to the total MB initially present is very small. Thus, although P does inhibit the MB adsorption, most of the MB adsorption cannot be inhibited by P. This is not surprising because charcoal is probably the most heterogeneous adsorbent known. It is quite probable, therefore, that MB adsorbs onto more than one type locus in charcoal. The fact that most of the MB adsorption is not inhibited by P means that P can inhibit the adsorption of MB only at one (or very few) of the type loci at which it is capable of adsorption in the absence of P. The per cent of the MB adsorbed which is resistant to P action can be calculated; this amount of MB should be subtracted from the amount of MB required for saturation (original concentration of MB—control value), then the per cent inhibition of MB adsorption values will become spread out over the whole range of inhibition from 0 to 100 per cent.

Since 0.7 ml. of a 1.0 per cent solution of MB was diluted to a final volume of 10 ml. in each flask, the MB concentration before adsorption by the charcoal was 0.070 per cent. The average concentration of MB found in the filtrates without P was approximately  $5 \times 10^{-5}$  per cent or less than 0.1 per cent of the total MB. At maximum inhibition by  $P^1$  the average concentration of MB in the filtrates in excess of the respective controls was approximately  $1 \times 10^{-3}$  per

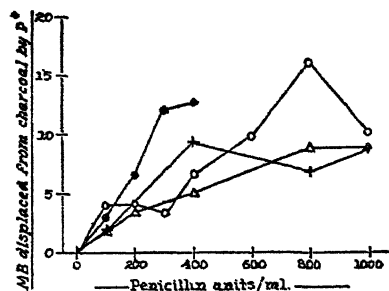


FIG. 1

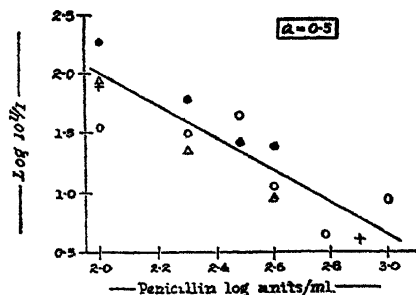


FIG. 2

FIG. 1. Inhibition of MB adsorption onto activated charcoal. Each type of symbol represents one experiment.

\* Ordinate units =  $\frac{\text{MB concentration in presence of } P_x}{\text{MB concentration in absence of } P}$

$P_x$  = any inhibitory concentration of P less than that which produces maximum inhibition.

FIG. 2. Mass law test of penicillin adsorption onto charcoal. Symbols as in Fig. 1.  $U$  = uninhibited MB adsorption.  $I$  = inhibited MB adsorption.

cent or about 1.4 per cent of the total MB. Almost 99 per cent of the total MB was still adsorbed, its adsorption apparently resistant to P. Thus if  $P_x$  represents any inhibitory concentration of P less than that which produces maximum inhibition,

$$(1) \quad \frac{(\text{MB concentration at } P_x \text{ concentration} - \text{control MB concentration})(100)}{(\text{MB concentration at maximum P inhibition} - \text{control MB concentration})}$$

= per cent inhibition of that fraction of adsorbed MB which is sensitive to P

<sup>1</sup> It is possible that by increasing the P concentration above the range employed here, further inhibition may be obtained above this first maximum seen in Fig. 1, but limited supplies of penicillin prevented testing this. If such were the case, there would be two possibilities: first, the P could be now adsorbing onto the same locus by a different type combination; second, the P could be now adsorbing onto a different locus in the charcoal, either by a similar or dissimilar type combination.

If we let  $C$  = the locus in charcoal which adsorbs MB and/or P

P = penicillin

$a$  = number of molecules of P adsorbing per unit of  $C$ ,

a test of whether or not P adsorbs at this locus  $C$  according to the law of mass action can be made as follows:

$$(2) \quad aP + C = P_aC$$

$$(3) \quad \frac{(P)^a(C)}{(P_aC)} = K$$

If we assume that  $(P_aC) \propto$  inhibition of MB adsorption =  $I$

then  $(C) \propto$  uninhibited MB adsorption =  $U$

Substituting  $I$  and  $U$  for their corresponding terms in Equation 3, and putting it into logarithmic form:

$$(4) \quad \log \frac{U}{I} = \log K - a \log (P)$$

If the adsorption occurs at one type locus and obeys the mass law, then plotting  $\log U/I$  versus  $\log (P)$  will give a straight line with a slope of  $a$ . The adsorption data of inhibition of MB adsorption by P are plotted in this way in Fig. 2 and it is seen that a straight line is approximated.

#### SUMMARY

Penicillin inhibits the adsorption of methylene blue onto activated charcoal in concentrations as low as 100 units/ml.

Penicillin in the low inhibitory concentration of 100 units/ml. antagonizes to a small extent the strong inhibition of MB adsorption by 0.1, 0.2, and 1 per cent peptone.

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# IMMUNOCHEMICAL PROPERTIES OF NATIVE AND DENATURED HORSE SERUM GLOBULINS\*†

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The problem of the relation of antibodies to normal serum globulins has been approached in the past by comparison of their chemical, physical, and immunological properties in the native state (*cf.* 1-3). Little information is available, however, on their relative behavior in the denatured state. This paper describes an investigation of the effects of denaturation by guanidine hydrochloride on certain physicochemical and immunological properties of Type I antipneumococcal horse serum globulin. In the light of recent theories of the structure and synthesis of antibodies (4, 5) it appeared also of interest to determine whether denaturation of normal and immune globulin might accentuate or diminish the antigenic relations which exist between them in the native form.

Comparative physicochemical properties of normal and immune serum globulins have been reviewed by Kabat (3). Wright (6) has reported that the antigenic nature of horse antibody proteins is essentially the same as that of horse normal gamma globulin. However, quantitative investigations by Treffers *et al.* (7-9) based on earlier findings of Ando *et al.* (10), and Marrack and Duff (11), revealed that antipneumococcal horse serum globulin differs from normal gamma globulin in its reaction with an antispecific precipitate rabbit serum. A high degree of cross-reactivity existed, and it was further noted that those antibodies that were precipitated by either antigen were incapable of differentiating between the two. These investigations provided experimental proof for earlier suggestions (12) that the groups on the antibody molecule responsible for antibody activity are unrelated to those involved in antigenic specificity.

Previous work on the effects of denaturation on the serological and physicochemical behavior of immune globulins has been extensively reviewed (1, 13, 14). The observation that monolayers of Types I and III antipneumococcal rabbit globulin are serologically active (15), suggests the independence of serological activity of a unique stereochemical configuration. However, in the light of a recent hypothesis (16) that formation of protein films involves the separation of preformed layers of peptide chains rather than unfolding into individual chains, a functional relation between intact *two dimensional* configurations and antibody activity is not excluded.

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† Preliminary data have been published in *Science*, 1943, **98**, 284.



While Pappenheimer, Lundgren, and Williams (17) reported treatment of diphtheria antitoxin with strong urea solutions to be without effect on its antitoxic properties, Wright (18) presented evidence for the loss of nearly half of its activity after denaturation by 7.5 M urea.

In a preceding publication (19) it was shown that the denaturing action of urea on normal horse serum globulin is greatly exceeded by that of guanidine hydrochloride, which gives rise to dissociation of the protein into halves as "unfolding" occurs. Accordingly, in order to accentuate the effects of denaturation on antibody properties, guanidine hydrochloride rather than urea was used in the present work.

## I. EXPERIMENTAL

### *Preparation of Materials*

*Normal Pseudoglobulin GI.*—This protein was prepared from normal horse serum as described previously (20). The purified material was monodisperse in diffusion (20) and electrophoresis (21).

*Type I Antipneumococcal Horse Globulin.*—The source of antibody globulin was a concentrate of antipneumococcal horse serum Types I and II, with a potency of 2000 mouse protective units of Type I, and 1750 mouse protective units of Type II, per cc.<sup>1</sup>

The method of preparation of the concentrate as given in a private communication by the Refining Department of the Lederle Laboratories is as follows:—

"The serum was diluted with one half of its volume of water, treated with urotropin, and then carbonated at 4 lbs. pressure over night. It was then diluted with three volumes of distilled water and allowed to settle in the chill-room over night. On the following day, the supernatant liquid was syphoned off and discarded. The precipitate was acidified to pH 5.4 (methyl red), and sufficient NaCl was added to make the concentration N/40. The material was again allowed to stand in the chill-room over night to permit the acid protein fraction to settle. The following day, the supernatant was syphoned off, neutralized to pH 7.4, and diluted with two volumes of water. After settling over night, the precipitate was concentrated by centrifugation and dissolved in 1 per cent salt. It was preserved with 0.25 per cent phenol and 1:25,000 phenyl mercuric acetate."

Type I antibody was isolated from this concentrate by specific precipitation with Type I polysaccharide (SI)<sup>1</sup> in the antibody excess region. Two hundred cc. batches of the concentrate were diluted to 900 cc. with saline, and six to seven consecutive precipitations carried out with decreasing amounts of carbohydrate. The floccules were washed five to seven times with 0.9 per cent NaCl, and the antibody recovered with 15 per cent NaCl as described by Heidelberger and Kendall (22). The antibody protein was precipitated from this extract by half-saturation with ammonium sulfate at pH 6.4, collected by centrifugation, dissolved in a minimum

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<sup>1</sup> We are indebted to Dr. W. G. Malcolm of the Lederle Laboratories, Inc. for a supply of these materials.

amount of 0.9 per cent NaCl, and dialyzed against the NaCl solution until free of ammonia. The final preparation contained 73 to 85 per cent specifically precipitable protein N.

Another batch of antibody was similarly prepared from Types I and II anti-pneumococcal horse serum (Lederle horse serum 4616, containing 400 mouse protective units of Type I and 650 mouse protective units of Type II per cc.).

*Type I Polysaccharide (SI).*—This was obtained through the courtesy of the Lederle Laboratories.<sup>1</sup> A stock solution was prepared by drying the solid material in a desiccator over Drierite for 24 hours, weighing out 10 mg. on a micro balance, and dissolving it in 0.9 per cent NaCl to a volume of 50 cc.

*Guanidine hydrochloride* was prepared from crude guanidine carbonate (American Cyanamid Company) by the method of Anson (23). It was recrystallized twice from anhydrous methanol by precipitation with anhydrous ether (24). Aqueous solutions of the final product had a pH of 6.8–7.0.

*Denatured and Regenerated Antibody.*—Denaturation of the antibody in 2 per cent solution was effected by 8 M guanidine hydrochloride in the presence of 0.9 per cent NaCl. After standing for 24 to 36 hours at room temperature, the solution was dialyzed against successive portions of 0.9 per cent NaCl, in the cold, until free of guanidine hydrochloride. Eighty-five to 90 per cent of the total protein was converted into an insoluble, irreversibly denatured form. The pH of the supernatant was found to be 6.5, with no additional precipitation occurring upon adjustment to pH 7.0. The quantitative distribution between irreversibly denatured and regenerated antibody was of the same order as that previously found for normal horse serum globulin (19).

The protein contained in the supernatant solution (regenerated fraction) was concentrated by precipitating at one-half saturation with ammonium sulfate, collecting at the centrifuge, dissolving in a minimum volume of 0.9 per cent NaCl, and dialyzing against salt solution until free of ammonia.

The white precipitate of irreversibly denatured antibody was washed twice with copious portions of 0.9 per cent NaCl. An extensive search for a suitable solvent found it to be highly insoluble in most of the common buffers and salts at pH 7.0. It was found to be soluble, however, in a solution containing 2 per cent NaCNS and 0.9 per cent NaCl, to the extent of about 0.3 mg. of protein N per cc.

## II. PHYSICOCHEMICAL MEASUREMENTS

The effects of denaturation and regeneration on the molecular properties of the antibody were investigated by measurements of viscosity, diffusion, and sedimentation in the ultracentrifuge.

### *Methods*

*Viscosity.*—Measurements were carried out in modified Ostwald viscometers at 25°C., as described previously (20).

*Diffusion.*—The refractometric scale method, described in detail elsewhere (25), was used for the determination of diffusion constants and for an estimation of the degree of monodispersity. Calculations were made with equations 10, 11, 12, and 14

of reference 25, and equation 1 of reference 26. The protein concentration was 4 mg. per cc., the solvent a 0.05 M acetate buffer, pH 5.5, containing also 0.2 M NaCl in addition to any reagent whose effect on the protein was to be studied.

*Sedimentation.*—Ultracentrifugal analyses were carried out by Dr. Max A. Lauffer at The Rockefeller Institute for Medical Research, Princeton, New Jersey, with the air-driven centrifuge (27). The optical system of Svensson was used for an estimation of the number and relative amounts of sedimenting components. Sedimentation constants were corrected to water at 20°C. and are expressed in Svedberg units (Table I).

TABLE I  
*Sedimentation Analyses of Native and Regenerated Type I Pneumococcal Horse Antibody*

Preparation	Concentration	$S_{H_2O}^{20}$	Remark
	mg. per cc.		
Native in pH 5.5 buffer*	10	14.9, 8.7	Heavy/light component = 1/4
	5	19.8, 7.1	Heavy/light component = 3/2
Native in pH 5.5 buffer* + 2 per cent NaCNS	4	6.8	Single boundary
Regenerated from 2 per cent NaCNS in pH 5.5 buffer*	11	14.5, 8.5	Even distribution between heavy and light components
	5.5	18.9, 6.9	
Regenerated from 7.5 M guanidine hydro- chloride, in pH 5.5 buffer*	5	14.7	Very diffuse boundary

\* Containing 0.05 M acetate and 0.2 M NaCl.

### Results

*Native Antibody.*—The sedimentation data given in Table I are in good accord with the values found by Heidelberger and Pedersen (28), Kabat (29), and Petermann and Pappenheimer (30). Thus the sedimentation constant of  $S = 19$  corresponds to the component with a molecular weight of about 990,000, whereas the lower sedimentation constant of  $S = 8.7$  may be identified as the component with a molecular weight of about 170,000. It is of interest to note that upon dilution the relative amount of the heavy component increases, as was previously noted by Heidelberger and Pedersen (28).

Diffusion measurements revealed the solutions to be polydisperse; the mean diffusion constant, as calculated from the standard deviation of the skewed curves, increased with time from  $1.0 \times 10^{-7}$  sq. cm./sec. to  $2.3 \times 10^{-7}$ . This shift is prob-

ably due to a diminution of the extent of mutual interaction between the large and asymmetric antibody molecules (25).

*Effect of Guanidine Hydrochloride.*—The influence of guanidine hydrochloride on the state of dispersion of the antibody was studied in concentrations of 2 M and 6 M, in analogy to similar experiments with normal horse serum globulin (19). There, it will be recalled, 2 M solutions of the salt caused aggregation of the globulin molecules in pairs, whereas in 6 M solutions the molecules were split into halves as partial unfolding occurred.

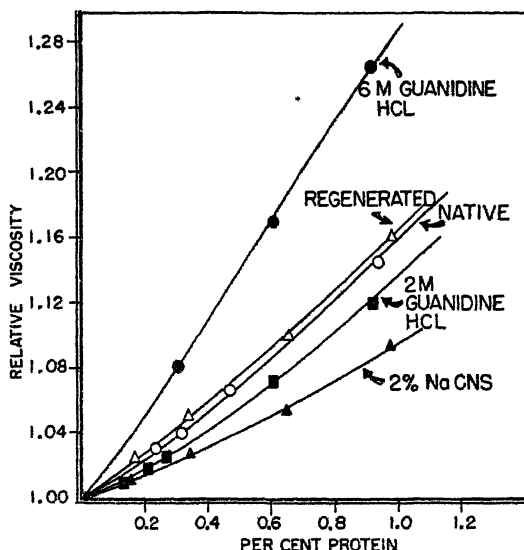


FIG. 1. Relative viscosities of native, denatured, and regenerated Type I anti-pneumococcal horse globulins plotted against protein concentration in weight per cent. The curves refer to, respectively, native antibody (O), native antibody in 2 per cent NaCNS ( $\blacktriangle$ ), denatured antibody in 2 M guanidine hydrochloride ( $\blacksquare$ ), denatured antibody in 6 M guanidine hydrochloride ( $\bullet$ ), and antibody regenerated from 8 M guanidine hydrochloride ( $\triangle$ ).

If the limiting slopes of the curves relating the relative viscosity to protein weight concentration are taken as a measure of the asymmetry of the protein molecules (20), the relative positions of the viscosity curves shown in Fig. 1 reveal a lower degree of asymmetry of the antibody in 2 M guanidine hydrochloride as compared to the native protein, whereas in 6 M solutions of this salt, the asymmetry is considerably higher. The limiting values of the weight intrinsic viscosities ( $\frac{\eta_{sp}}{c}$ ) were 12.7, 8.3, and 25.5 for native antibody, and

antibody dissolved in 2 M and 6 M guanidine hydrochloride, respectively (Table II).

It has not been possible to estimate from viscosity and diffusion data the molecular weight of the antibody in 2 M guanidine hydrochloride, since diffusion measurements revealed the solutions to contain more than one component. Calculations with equations 10, 12 (25), and 1 (26) yielded a mean value of  $2.62 \times 10^{-7}$  with a standard deviation of  $\pm 0.032$ , whereas with equation 13 (25) the calculated mean diffusion constant was considerably higher; *i.e.*,  $4.5 \times 10^{-7}$ .

In the presence of 6 M guanidine hydrochloride, the diffusion pattern was indicative of monodispersity, all four methods of calculation yielding consistent values. Converted to normal coordinates, the experimental diffusion curves were superimposable on a Gaussian distribution curve. The mean diffusion constant was  $2.88 \times 10^{-7} \pm 0.19$ , which, with the empirical correc-

TABLE II

*Intrinsic Viscosities ( $\frac{\eta_{sp}}{c}$ ) of Native and Denatured Type I Pneumococcal Horse Antibodies\**

Preparation	Intrinsic viscosity
Native.....	12.7
Native in 2 per cent NaCNS.....	8.5
Denatured in 2 M guanidine hydrochloride.....	8.3
Denatured in 6 M guanidine hydrochloride.....	25.5
Regenerated from 8 M guanidine hydrochloride.....	14.4

\* All measurements were performed in solutions containing 0.05 M acetate buffer pH 5.5 and 0.2 M NaCl.

tion factor for solvent viscosity (31), and in combination with the viscosity data, yields a molecular weight of about 170,000. However, the influence of guanidine hydrochloride cannot be ascribed to a mere disaggregation of the native antibody into subunits corresponding to normal globulin (9) since the apparent molecular asymmetry is considerably higher. Expressed in terms of a ratio of major to minor axis of a prolate ellipsoid of revolution, and assuming 30 per cent of hydration, a value of 15.0 is calculated as compared to 5.2 for normal globulin. Size and shape properties of antibody denatured by guanidine hydrochloride are similar to those of urea-denatured normal globulin.

*Regenerated Antibody.*—An antibody preparation, regenerated after denaturation by 8 M guanidine hydrochloride as described above (page 423) was subjected to viscosity and diffusion measurements. As shown in Fig. 1 and Table II, the relative viscosity resembles closely that of the native protein. Diffusion measurements indicated the material to be aggregated to a considerable extent, the mean value calculated with equations 10, 12 (25), and 1 (26) being

$0.94 \times 10^{-7}$ . This lack of monodispersity precluded estimations of molecular weights.

*Effect of NaCNS.*—The relative insolubility of the irreversibly denatured antibody in common buffer solutions necessitated recourse to the solvent action of NaCNS, as described in section I (page 423). Since the comparative serological activities of native and irreversibly denatured antibody were determined in a medium containing 2 per cent NaCNS in addition to 0.9 per cent NaCl, it was of interest to investigate the effect of NaCNS on the molecular properties of the native antibody. Sedimentation analyses recorded in Table I revealed the presence of a single boundary with a sedimentation constant of  $S = 6.8$ ; *i.e.*, approximately that of the light globulin component of antisera. This value, in conjunction with the limiting intrinsic weight viscosity of 8.5, yields a molecular weight of about 140,000 and an axial ratio of  $b/a = 9.0$ . Accordingly, in the presence of NaCNS the larger antibody molecules appear to become disaggregated into components approximating to some degree the size and shape properties of *native*, normal horse serum globulin.

In order to determine whether the disaggregating effect of NaCNS can be reversed, a 1.5 per cent solution of the native antibody was dissolved in 0.9 per cent NaCl, containing 2 per cent NaCNS. After 48 hours' standing, NaCNS was removed by dialysis against acetate-saline buffer, and the solution again subjected to ultracentrifugal analysis. The data given in Table I reveal practically complete reversal to the state of dispersion of the original preparation, with about even distribution between the heavy ( $S = 18.9$ ) and light ( $S = 6.9$ ) components. This finding is of considerable importance for the interpretation of the noted effects of NaCNS on the course of the precipitin reaction, as discussed in section III (page 430).

### III. GLOBULIN AS ANTIBODY

#### *Methods*

The quantitative method of Heidelberger and Kendall (32) was used to determine the amount of specifically precipitable protein N and combining ratios. The amounts of antibody solution used were such as to contain a total of 0.3 to 0.8 mg. of precipitable N. All determinations were carried out in duplicate, the pH of the solutions being maintained within a range of 6.4–7.4. The supernatants were tested for excess antigen or antibody, the symbols + (flocculation),  $\pm$  (turbidity), and 0 (no visible change) denoting the appearance of the supernatant 1 hour after the addition of antigen or antibody. For these tests the dose of antigen was the minimum amount giving visible flocculation; *i.e.*, about 6 to 28  $\gamma$ .

#### *Results*

Qualitative precipitin tests revealed both the irreversibly denatured and the regenerated antibody to react strongly with SI. This was true not only of the fractions derived from the concentrate but also of those obtained by denatura-

tion and regeneration of whole antipneumococcal horse serum (No. 4616). The latter finding is of importance since it indicates that the serological activity of the regenerated antibody is not due to the adventitious presence of polysaccharide which might have directed the regeneration process toward the reformation of native antibody (33). Since the regenerated whole serum (No. 4616) also reacted strongly with SII, the reactivity of this fraction cannot be ascribed to a unique property of Type I antipneumococcal globulin.

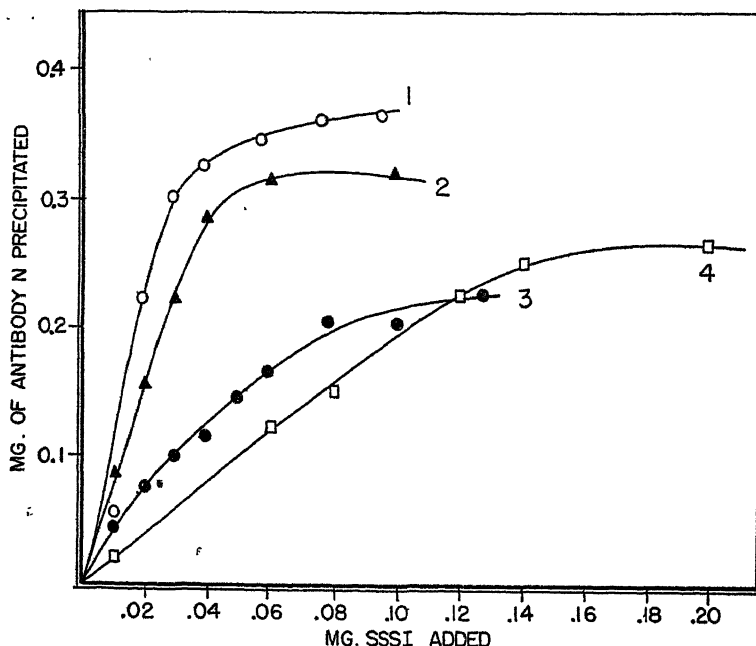


FIG. 2. Quantitative absorption of native, irreversibly denatured, and regenerated type I antipneumococcal horse antibodies by SI. All curves are reduced from the experimental data to the same content of total protein N (0.44 mg.). Curve 1 refers to regenerated antibody in 0.9 per cent NaCl, curve 2 to native antibody in 0.9 per cent NaCl, curve 3 to native antibody in 2 per cent NaCNS + 0.9 per cent NaCl, and curve 4 to irreversibly denatured antibody in 2 per cent NaCNS + 0.9 per cent NaCl.

The course of quantitative precipitin titrations of native, denatured, and regenerated antibody with SI is given in Table III and plotted in Fig. 2. The relative insolubility of the irreversibly denatured fraction required the serological activity of the latter to be compared with that of the native antibody in the presence of 2 per cent NaCNS.

It is apparent from the positions of the curves, reduced in Fig. 2 to the same

TABLE III  
*Quantitative Absorption of Antibody Solutions with SI*

SI added	Antibody N precipitated	$\frac{\text{Antibody N}}{\text{SI}}$	Supernatant tested for	
			Excess antibody	Excess antigen
mg.	mg.			
Native antibody in 0.9 per cent NaCl				
0.02	0.173	8.65	+	0
0.04	0.319	8.0	+	0
0.06	0.455	7.6	+	0
0.08	0.587	7.35	+	0
0.12	0.641	5.35	+	0
0.20	0.651	3.26	±	0
0.24	0.772	—	0	+
		$R^* = 4.65$		
72 per cent specifically precipitable				
Regenerated antibody in 0.9 per cent NaCl				
0.02	0.121†	—	+	0
0.04	0.471	11.8	+	0
0.06	0.642	10.7	+	0
0.08	0.697	8.6	+	0
0.12	0.739	6.2	+	0
0.16	0.774	4.8	±	0
0.20	0.780	—	0	+
		$R^* = 7.3$		
83 per cent specifically precipitable				
Native antibody in 2 per cent NaCNS				
0.02	0.091	4.6	+	0
0.04	0.156	3.9	+	0
0.06	0.205	3.4	+	0
0.08	0.236	3.0	+	0
0.10	0.299	3.0	+	0
0.12	0.342	2.9	+	0
0.16	0.421	2.6	+	0
0.20	0.415	2.1	±	0
0.26	0.421	1.7	?	?
49 per cent specifically precipitable				
Irreversibly denatured antibody in 2 per cent NaCNS				
0.01	0.021	2.10	+	0
0.06	0.123	2.05	+	0
0.08	0.152	1.90	+	0
0.12	0.225	1.87	+	0
0.14	0.249	1.78	?	0
0.24	0.253	1.00	0	+
58 per cent specifically precipitable				

\*  $R = \frac{\text{Antibody N}}{\text{SI}}$  at equivalence.

† Incomplete separation despite prolonged centrifugation.



total protein N content of 0.44 mg., that the regenerated antibody is less active than the native in that, gram for gram, it combines with less antigen. The optimal combining weight ratios, calculated from the equation (34)

$$\frac{N}{S} = 2R - \frac{R^2 S}{A} \quad (1)$$

are 4.6 and 7.8, for native and regenerated antibody, respectively. The extent of specific precipitation, however, was about the same for the two fractions; *i.e.*, 72 and 83 per cent.

Titration data of the irreversibly denatured fraction do not yield readily to comparative analysis since the presence of NaCNS appears to have a profound effect on the course of the reaction. The relation of antibody precipitated to antigen added follows neither of the equations of Heidelberger and Kendall and appears to defy quantitative interpretation. Thus, equation (1) yields for the native antibody in 2 per cent NaCNS a value of 0.248 for *A* whereas the actual value appears to be about 0.4 mg. With the irreversibly denatured antibody, the calculated value of *A* is higher than that found; *i.e.*, 0.43 as compared to 0.25. These discrepancies are not diminished when the empirical equation (35)

$$\frac{N}{S} = k_1 - k_2 S^{1/2} \quad (2)$$

is applied. Interpolation of the plotted data yields approximate combining weight ratios at the beginning of the equivalence zone of 2.6 and 1.8 for native and irreversibly denatured antibody, respectively. The corresponding values for per cent specifically precipitable N are 49 and 58. Assuming the influence of NaCNS on the course of the titration to be the same for both antibody fractions, it follows that, per unit weight, the irreversibly denatured fraction is serologically more active than the native and that both fractions are precipitable to the same extent.

In order to show that the specific serological properties of the irreversibly denatured fraction were not due to the incomplete removal of regenerated material, the twice washed precipitate obtained following removal of guanidine hydrochloride (page 423), was subjected to three consecutive extractions with a solution containing 2 per cent NaCNS in 0.9 per cent NaCl. Although each successive extraction yielded less protein than the preceding one, quantitative precipitin titration with SI revealed the per cent specifically precipitable protein N, and combining ratio at the beginning of the equivalence zone, to be the same in each case.

*Effect of NaCNS.*—The inhibitory effect of NaCNS on the precipitin titration is revealed by (a) a decreased specific precipitation (compare the data for native anti-

body in the presence and absence of NaCNS); (b) a departure from the normal course of the reaction; and (c) a lowering of the combining weight ratio at the equivalence point. This behavior is analogous, in part, to that observed by Heidelberger, Kendall, and Teorell (36), for the influence of high concentrations of NaCl on the reaction of SIII with the homologous antibody, although there, in contrast to the present data, the general course of the reaction was not altered. In neither instance was the effect of salts directed toward an increased solubility of the precipitate since no additional precipitation occurred upon dialysis unless more antigen was added. This is in contrast to sodium salicylate which appears merely to increase the solubility, by way of its action on the antibody (37). While ultracentrifugal analysis revealed the heavy antibody component to be split under the influence of NaCNS, yielding a remarkably homogeneous solution with a sedimentation constant approximating that of normal globulin, it is unlikely that this is the factor responsible for inhibition of precipitation. It has been shown that pneumococcal horse antibodies may be associated with globulin components varying from  $S = 19$  all the way down to  $S = 7$  and that this graduation in size and shape is unrelated to serological activity (29, 30). Further, the dissociation of the antibodies by NaCNS is reversible in a remarkable fashion since removal of the salt restores the sedimentation characteristics of the original material. Accordingly, the degree of denaturation incurred in this reaction must have been associated with only minor changes in protein structure (14).

Heidelberger *et al.* have suggested that competition of the *cation* with the antibody for combination with the acidic groups of the polysaccharide is responsible for the inhibitory effect of concentrated NaCl (36). Undoubtedly this does not apply to the present phenomenon since it is an *anionic* effect, and hence it can hardly be explained in terms of an electrostatic shielding of these groups. However, the general hypothesis of a shift of the equilibrium between free and combined antibody in the direction of the former has much to recommend itself, although the available data fail to elucidate the underlying mechanism. While an excess of antigen might serve to attain nearly complete combination, even in the presence of NaCNS, the increased solubility of the complex in the region of antigen excess prevents additional precipitation, unless NaCNS is first removed by dialysis.

*Comparison of the Serological Properties of the Fractions.*—Since precipitin titrations were carried out with the same antigen (haptene), variations in combining weight ratios and degrees of specific precipitation may be ascribed entirely to modifications in the structure of the antibody.

The observed differences in optimal combining weight ratios of native and regenerated antibody (Table III) may be related to changes in molecular weight and shape, to changes in effective antibody valence, or to both. For the molar combining ratio,  $R_M$ , to remain constant, at constant antibody valence, the combining weight ratio,  $R$ , should increase in proportion to the increase in molecular weight of the antibody,  $M_A$ . According to the relation

$$R_M = R \frac{M_B}{M_A} \quad (3)$$

an increase in  $R$  from 4.6 to 7.3 may be ascribed to a 1.6-fold increase in molecular weight, while by the same reasoning the molecular weight of the irreversibly denatured antibody is about two-thirds that of the native.

The fact that antipneumococcal horse antibody ( $M = 990,000$ ) combines with SI in a higher ratio than does pneumococcal rabbit antibody ( $M = 150,000$ ) is in keeping with this relation, as may be the noted increase in combining weight ratio, following acidification of rabbit antipneumococcal serum (38). Likewise, pepsin-treated diphtheria antitoxin ( $M = 113,000$ ) combines with twice as much toxin (39) as does the untreated antitoxin ( $M = 170,000$ ). Preliminary data of Petermann and Pappenheimer (30) have shown that digested pneumococcus horse antibody ( $S_{5.2}$ ) also combines with twice as much polysaccharide as the native, regardless of the molecular weight of the starting material.

Another explanation, readily compatible with the general physical concept of the denaturation and regeneration processes, may be found in variations of the effective antibody valence. The native antibody has upon its surface a number of reactive sites to which its serological activity may be attributed. If the influence of multivalent antigen (40) on the *in vivo* synthesis of antibody (4, 5) becomes effective before the polypeptide chains are molded into a specific three-dimensional configuration, it may be assumed that additional serologically active centers are present within the molecule.<sup>2</sup> Upon denaturation, the molecule splits, and unfolds to a certain extent, resulting in the liberation of some of these internal reactive groups. Experimental evidence for the unmasking of groups upon denaturation may be found in the observations that protein sulfhydryl groups that are either not detectable in the native state (egg albumin, excelsin, etc.) or detectable only in part (myosin, urease), become exposed to varying degrees upon denaturation by heat, urea, or guanidine hydrochloride (14). It has been shown also that disulfide groups detectable after denaturation of serum albumin by heat or urea revert to the unreactive state after regeneration (14).

The serological activity of the denatured and regenerated antibody fractions, respectively, parallels to a considerable measure the reactivity of the protein sulfhydryl and disulfide groups. The decreased combining weight ratio of the irreversibly denatured antibody suggests the availability of extra serologically active groups and may be ascribed to an incomplete recoiling of the polypeptide chains, followed by aggregation, after removal of guanidine hydrochloride. Combination with the antigen occurs by means of the latter's penetration into the interstices of the loose aggregates. The higher combining

<sup>2</sup> Such an assumption is compatible with both, the older template hypothesis of Haurowitz and Mudd (2) as well as with the newer ideas of Burnet (5) who suggests that antibody formation results from a specific modification of the intracellular proteinases, initiated by the antigen.

weight ratio of the regenerated fraction would suggest that some of the originally available groups of the native antibody have become masked during the regeneration process.

The most important conclusion derived from the present data is that the denatured antibody is serologically active, and that, discounting a specific effect of NaCNS on either one of the fractions, the extent of specific precipitation is of the same order of magnitude for the native and irreversibly denatured proteins.

#### IV. GLOBULINS AS ANTIGENS

In order to determine whether denaturation and regeneration of the antibody resulted in significant changes in antigenic structure, or in its antigenic relation to normal globulin, rabbit antisera to native and denatured antibody globulin and normal globulin, respectively, were prepared and subjected to quantitative precipitin titrations with homologous and heterologous antigens. Previous work (7-11) has already demonstrated the conducibility of such an experimental approach.

##### *Methods*

*Antigens.*—The antigens employed were: (1) native antibody (NA), (2) irreversibly denatured antibody (DA), (3) native normal horse serum globulin GI (NG), and (4) normal GI irreversibly denatured by 8 M guanidine hydrochloride (DG). The preparation of NA and DA is described in section I of this paper, that of NG and DG in preceding publications (19, 20). NA and NG were dissolved in Ringer-phosphate solution, pH 7.4; DG was dissolved in a slight excess of alkali and then dialyzed against the Ringer-phosphate solution, whereas DA was used as a suspension in 0.9 M NaCl.

*Antisera.*—Four groups of 12 rabbits each (1.8 to 3.0 kilos body weight) were given increasing doses of the four antigens, intravenously, thrice weekly, over a period of 2 weeks. The total dose was 20 mg. per kilo body weight. Later in the course of investigation, stronger antisera were prepared by immunization with larger doses (75 mg.) over a period of 3 weeks. The rabbits were bled by heart puncture 8 to 10 days after the last administration. The blood was allowed to clot in paraffined tubes, the sera collected, cleared by centrifugation, and stored in 1:5000 merthiolate. Control sera, obtained from each rabbit prior to immunization, proved to be entirely negative.

*Precipitin Titrations.*—For comparing the relative strength of the various antisera, the serum dilution method of Martin (41) was employed, using antigen solutions containing 6 to 28  $\gamma$  of protein per cc. The quantitative precipitin method of Heidelberger and Kendall (32) was used to determine the amount of precipitable antibody N and combining ratios of the pooled sera obtained from each of the four groups of antisera. For individual precipitations, the amount of serum was such as to yield 0.3 to 0.8 mg. of protein N specifically precipitable by the homologous antigen. All determinations were performed in duplicate. The supernatant solutions were

tested for excess antigen, and for antibodies to the homologous and heterologous antigens. The symbols ++ (flocculation); + (heavy turbidity), ± (slight turbidity), ? (doubtful), and 0 (no visible change) denote the appearance of the supernatant 1 hour after the addition of antigen or antiserum.

### Results

*Antigenicity.*—Previous work from this laboratory revealed that denaturation of horse serum albumin by urea (42), and of bovine albumin by guanidine hydrochloride (43) resulted in a significant decrease in the ability of the protein to elicit antibody formation in the rabbit. The results of similar experiments with normal and irreversibly denatured normal globulin GI, and antibody globulin, respectively, are given in Table IV.

The second column in Table IV lists the titers, expressed as gamma antigen optimally combined per cubic centimeter of serum, whereas the third column refers to the antibody protein content in milligrams per cubic centimeter of serum. Precipitin titrations by the method of Martin (41), as employed here, yield a quantitative measure of the antibody content only if the optimum combining ratios are known. Thus the highest serum dilution showing the visible turbidity will be higher for antigens combining in lower ratios. Accordingly, the apparent difference between mean titers of native and denatured normal globulin, (see column 2 of Table IV) vanishes when the respective combining ratios of 4.8 and 2.8 are taken into account (see column 3). The spread in antibody content was so wide as to render the difference in mean titers statistically insignificant ( $p < 0.9 > 0.8$ ).<sup>3</sup>

Similar experiments with native and denatured antibody globulin (Table IV) found the denatured fraction to be more antigenic ( $p < 0.1 > 0.05$ ). This greater response to antibody formation by the denatured protein is compatible with the generally established fact that protein suspensions, as employed for the immunization with this fraction, are more potent antigens than are protein solutions (44), and accordingly, it need not be ascribed to the effects of denaturation.

The discrepancy between the present findings and those previously reported for serum albumin (42) requires explanation. The possibility was considered that the greater susceptibility of the denatured (regenerated) albumin to tryptic hydrolysis might lead to its more rapid destruction in the body and thereby might account for its lower antigenic activity. Supporting evidence for such a hypothesis has been provided recently by Haurowitz *et al.* (45). These workers coupled weakly antigenic gelatin and strongly antigenic sheep serum globulin with arsanilic acid and prepared rabbit antisera to these antigens. With the easily detectable arsenic as tracer, it was found that within 60 minutes after administration the rabbits immunized with

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<sup>3</sup>  $p$  is the probability that a given deviation from expectation (based upon hypothesis) shall occur by pure chance. Thus  $p = 0.8$  means that in 80 per cent of the total number of observations, an event will occur by mere coincidence.

TABLE IV

*Precipitin Titrations of Rabbit Antisera to Native and Denatured Normal and Antibody Globulin with Homologous Antigens*  
*Normal Horse Globulin GI*

<i>Anti-native</i>			<i>Anti-denatured</i>		
Rabbit	Titer*	Antibody protein	Rabbit	Titer*	Antibody protein
		<i>mg. per cc.</i>			<i>mg. per cc.</i>
1	224	1.08	13	480	1.34
2	448	2.15	14	480	1.34
3	112	0.54	15	480	1.34
4	224	1.08	16	960	2.68
5	112	0.54	17	240	0.67
6	896	4.30	18	480	1.34
7	224	1.08	19	480	1.34
8	112	0.54	20	960	2.68
9	448	2.15	21	480	1.34
10	56	0.27	22	240	0.67
11	448	2.15	23	480	1.34
			24	240	0.67
Average.....		1.44 ± 1.18	Average.....		1.50 ± 0.84

*Antibody Globulin*

<i>Anti-native</i>			<i>Anti-denatured†</i>		
Rabbit	Titer*	Antibody protein	Rabbit	Titer*	Antibody protein
		<i>mg. per cc.</i>			<i>mg. per cc.</i>
25	192	0.64	37	680	2.52
26	384	1.28	38	340	1.26
27	384	1.28	39	340	1.26
28	384	1.28	40	1360	5.04
29	48	0.16	41	340	1.26
30	192	0.64	42	340	1.26
31	96	0.32	43	170	0.63
32	96	0.32	44	170	0.63
33	96	0.32	45	1360	5.04
34	384	1.28			
Average.....		0.75 ± 0.48	Average .....		2.10 ± 1.75

\* Titer expressed as  $\gamma$  protein optimally bound by 1 cc. of serum.

† Titrated with native antibody.

the diazotized gelatin excreted in the urine over 60 per cent of the injected arsenic as compared to 9 per cent excreted during the same time by the animals to whom diazotized serum globulin had been administered.

The divergence of the antigenic behavior of serum albumin and serum globulin, respectively, is also compatible with the findings that the difference in rate of tryptic hydrolysis is smaller between native and denatured globulin than between the corresponding albumin fractions, and, furthermore, that the absolute rate of proteolysis is lower for denatured globulin than for native albumin (46).

*Specificity.*—Tables V to VIII and Figs. 3 to 6 show the results of quantitative precipitin titrations of the four groups of pooled antisera with homologous and heterologous antigens. From the positions of the curves it may be seen that, as a rule, the homologous antigens remove more antibody than do the heterologous ones, the two exceptions being the reaction of NG with anti-DG serum and of NA with anti-DA serum. These apparent discrepancies are discussed below. A cursory inspection of the data reveals distinct antigenic differences between the four antigens in that no two antigens react in exactly the same manner with a given antiserum. Also, in no instance after a serum was exhausted by absorption with the homologous antigen, did additional precipitation occur upon addition of heterologous antigen to the supernatant; however, when the heterologous antigen did not remove all of the precipitin, additional precipitation ensued when the homologous antigen was added to the supernatant solution (see Tables V to VIII).

From quantitative experiments with rabbit antibodies to a specific precipitate of pneumococcal horse antibodies, Treffers, Moore, and Heidelberger (9) concluded that while normal and immune globulin have different antigenic properties, "the reactivity of the normal horse gamma globulin *per mg. of antibody* precipitated is the same as that of the immune gamma globulin antigen" and "that the portion of the rabbit antibody which is precipitated by both globulins is incapable of differentiating between them." It is the object of the following discussion to interpret the present data in the light of these conclusions, and to determine whether denaturation of either antibody globulin or normal globulin by guanidine hydrochloride, has served to diminish the antigenic differences that exist between them in the native state. The present data include precipitin titrations of antibody globulin and normal globulin with antisera to both, and in this sense they go beyond the analyses of Treffers, Moore, and Heidelberger (9) which were limited to antisera to but one of them; *i.e.*, to a specific precipitate of pneumococcal horse antibody globulin.

Considering first the general course of the reactions, it was found that with the exception of DA which, as stated, was used as a suspension, the data fit closely the empirical equation (35)

$$\frac{N}{S} = k_1 - k_2 S^{1/2} \quad (2)$$

TABLE V  
Quantitative Absorption of Anti-NG Serum

Antigen N added	Antibody N precipitated*	Antibody N / Antigen N	Supernatant tested with			
mg.	mg.					
2 cc. of anti-NG absorbed by NG						
			NG	Anti-NG	DG	NA
0.01	0.109	10.9	++	0	+	0
0.02	0.176	8.8	++	0	+	0
0.04	0.289	7.2	+	0	+	0
0.08	(0.382)	(4.3)	±	+	0	0
0.10	(0.379)	—	0	+	0	0
0.12	(0.361)	—	0	+	0	0
2 cc. of anti-NG absorbed by NA						
			NA	Anti-NG	NG	DG
0.011	0.064	5.6	+	0	++	++
0.023	0.101	4.4	+	0	++	++
0.034	0.120	3.5	±	±	++	++
0.046	(0.136)	(2.8)	±	+	++	++
0.068	(0.140)	—	0	+	++	++
0.092	(0.143)	—	0	+	++	++
2 cc. of anti-NG absorbed by DG						
			DG	Anti-NG	NG	NA
0.012	0.078	6.5	+	0	+	+
0.024	0.129	5.4	+	0	+	+
0.048	0.179	3.7	+	0	+	+
0.072	0.230	3.2	+	0	+	?
0.096	0.257	2.7	±	±	+	0
0.120	(0.255)	(2.1)	±	+	+	0
2 cc. of anti-NG absorbed by DA suspension						
			NA	Anti-NG	NG	DG
0.036	0.053	—	+	0	++	++
0.072	0.080	—	+	0	++	++
0.108	0.113	—	+	0	++	++
0.144	0.128	—	±	0	++	++
0.216	0.145	—	±	0	++	++
0.324	0.165	—	0	0	++	++

\*Antibody N = total N precipitated—antigen N added.



TABLE VI  
*Quantitative Absorption of Anti-DG Serum*

Antigen N added	Antibody N precipitated*	Antibody N / Antigen N	Supernatant tested with			
mg.	mg.					
1 cc. of anti-DG absorbed by DG						
			DG	Anti-DG	NG	NA
0.012	0.060	5.0	+	0	+	+
0.024	0.116	4.8	+	0	+	+
0.036	0.132	3.7	+	0	(+)	±
0.048	0.137	2.9	±	±	±	0
0.072	(0.138)	(1.9)	0	+	0	0
1 cc. of anti-DG absorbed by NA						
			NA	Anti-DG	NG	DG
0.006	0.027	4.5	+	0	++	++
0.012	0.044	3.7	+	0	++	++
0.020	0.068	3.5	+	0	++	++
0.026	0.092	3.5	±	0	++	++
1 cc. of anti-DG absorbed by NG						
			NG	Anti-DG	DG	NA
0.01	0.068	6.8	+	0	+	+
0.02	0.118	5.9	+	0	+	+
0.03	0.145	4.9	+	0	+	±
0.04	0.162	4.1	±	±	+	0
0.06	(0.136)	(2.1)	0	+	+	0
1 cc. of anti-DG absorbed by DA suspension						
			DG	Anti-DG	NG	NA
0.012	0.023	—	++	0	++	++
0.024	0.043	—	++	0	++	++
0.036	0.045	—	++	0	++	++
0.060	0.073	—	++	0	++	++
0.084	0.092	—	++	0	++	++
0.120	0.108	—	++	0	++	+

\* Antibody N = total N precipitated—antigen N added.

TABLE VII  
*Quantitative Absorption of Anti-NA Serum*

Antigen N added	Antibody N precipitated*	Antibody N / Antigen N	Supernatant tested with			
mg.	mg.					
1.5 cc. of anti-NA absorbed by NA						
			NA	Anti-NA	NG	DG
0.023	0.156	6.8	+	0	0	0
0.046	0.256	5.6	+	0	0	0
0.068	0.333	4.9	+	0	0	0
0.091	0.356	4.0	+	0	0	0
0.125	(0.372)	(2.9)	?	+	0	0
0.160	(0.281)	—	0	+	0	0
0.193	—	—	0	+	0	0
1.5 cc. of anti-NA absorbed by NG						
			NG	Anti-NA	DG	NA
0.01	0.063	6.3	+	0	+	+
0.02	0.087	4.1	0	?	0	+
0.03	(0.091)	—	0	+	0	+
0.04	(0.086)	—	0	+	0	+
0.06	—	—	0	+	0	+
1.5 cc. of anti-NA absorbed by DG						
			DG	Anti-NA	NG	NA
0.012	0.046	3.85	+	0	+	+
0.024	0.063	2.6	+	0	+	+
0.036	0.071	1.95	0	±	0	+
0.048	(0.066)	—	0	+	0	+
1.5 cc. of anti-NA absorbed by DA suspension						
			NA	Anti-NA	NG	DG
0.108	0.108	—	++	0	+	+
0.216	0.175	—	+	0	±	?
0.324	0.212	—	+	0	0	0
0.432	0.243	—	+	0	0	0
0.648	0.279	—	±	0	0	0
0.864	(0.226)	—	0	0	0	0

\* Antibody N = total N—antigen N added.

TABLE VIII  
*Quantitative Absorption of Anti-DA Serum*

Antigen N added	Antibody N* precipitated	Antibody N / Antigen N	Supernatant tested with			
mg.	mg.					
2 cc. of anti-DA absorbed by DA suspension						
			NA	Anti-DA	NG	DG
0.108	0.125	—	+	0	±	±
0.216	0.217	—	+	0	0	0
0.324	0.297	—	+	0	0	0
0.432	0.335	—	+	0	0	0
0.648	0.410	—	±	0	0	0
0.864	(0.311)	—	0	0	0	0
2 cc. of anti-DA absorbed by NG						
			NG	Anti-DA	NA	DG
0.01	0.074	7.4	+	0	++	+
0.02	0.108	5.4	+	0	+	+
0.03	0.131	4.4	±	±	+	±
0.04	0.131	3.3	±	±	+	±
0.05	(0.124)	—	0	+	+	?
2 cc. of anti-DA absorbed by NA						
			NA	Anti-DA	NG	DG
0.023	0.179	7.9	+	0	0	±
0.046	0.269	5.8	+	0	0	±
0.068	0.375	5.5	+	0	0	0
0.091	0.423	4.6	+	0	0	0
0.114	0.448	3.9	±	0	0	0
0.136	0.465	(3.4)	±	±	0	0
0.171	0.464	—	?	+	0	0
2 cc. of anti-DA absorbed by DG						
			DG	Anti-DA	NA	NG
0.024	0.084	3.5	+	0	+	+
0.036	0.093	2.6	+	0	+	±
0.048	0.099	2.1	+	±	+	±
0.060	(0.112)	—	±	+	+	0

\* Antibody N = total N precipitated—antigen N added.

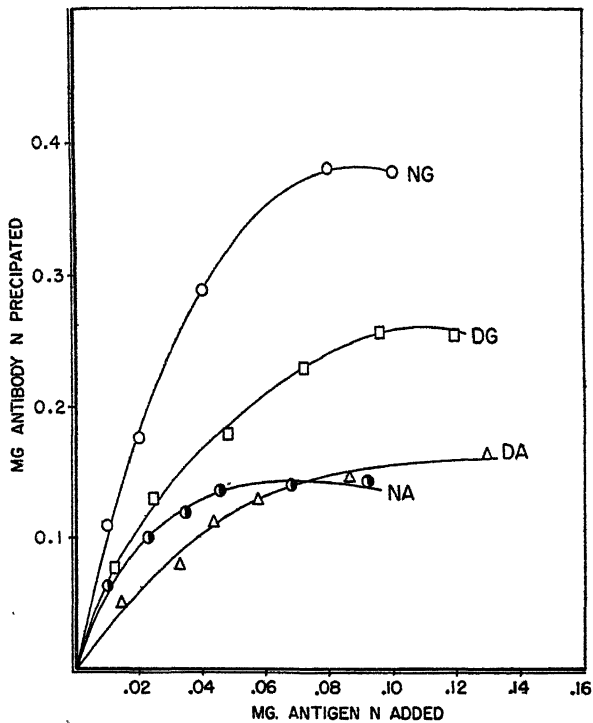


FIG. 3. Quantitative absorption of anti-NG rabbit serum by NG, DG, NA, and DA. Since greater quantities of DA suspension are needed to remove comparable amounts of antibody N, the experimental points for this curve have been brought into the graph by reducing their abscissae 2.5 times.

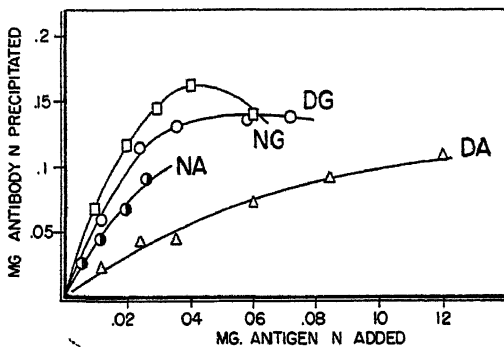


FIG. 4. Quantitative absorption of anti-DG rabbit serum by NG, DG, NA, and DA.

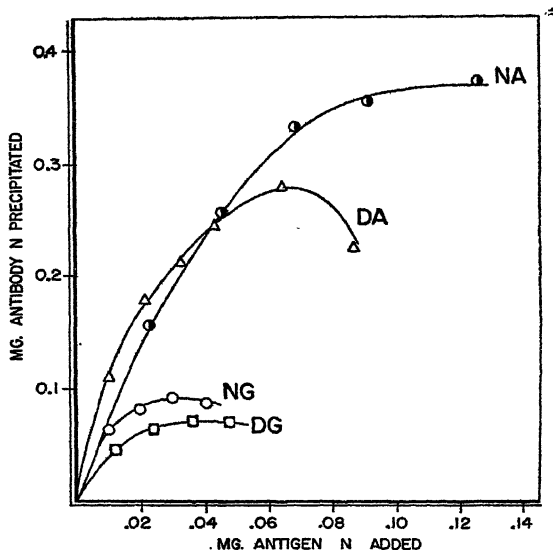


FIG. 5. Quantitative absorption of anti-NA rabbit serum by NG, DG, NA, and DA. Since greater quantities of DA suspension are needed to remove comparable amounts of antibody N, the experimental points for this curve have been brought into the graph by reducing their abscissae 10 times.

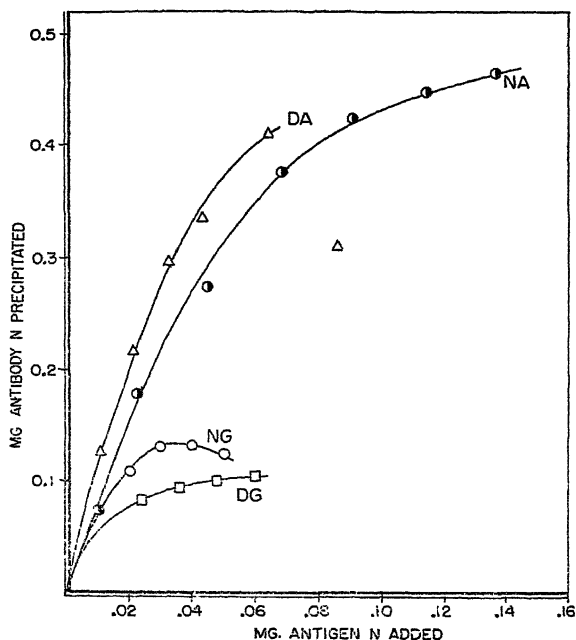


FIG. 6. Quantitative absorption of anti-DA rabbit serum by NG, DG, NA, and DA. Since greater quantities of DA suspension are needed to remove comparable amounts of antibody N, the experimental points for this curve have been brought into the graph by reducing their abscissae 10 times.

where  $N/S$  is the ratio of antibody  $N$  precipitated to antigen  $N$  added, and  $k_1$  and  $k_2$  are reaction constants. While this equation is empirical, the meaning of the reaction constants can be evaluated mathematically (35) by differentiation with respect to  $S$ , and setting  $dN/dS$  equal to zero. This yields

$$k_1 = 3R'' \quad (4)$$

$$k_2 = 2\sqrt{\frac{R''^2}{A}} \quad (5)$$

where  $R''$  is the optimal combining ratio at the equivalence point and  $A$  the total amount of specifically precipitable antibody  $N$ . In Table IX the calculated values for  $k_1$  and  $k_2$  are listed.<sup>4</sup> While  $k_1$  is independent of the total amount of antibody removed,  $k_2$  is not. Wherever reactions of two antigens with a given antiserum yield identical values of  $k_1$  it may be suspected that those antigenic groups which do react with the antibody are the same for the two antigens. In such a case, recalculation of the reaction equation to the same maximum amount of precipitable antibody,  $A$ , should yield also identical values for  $k_2$ . This procedure has been employed to advantage in several cross-reactive systems (such as the pneumococcal polysaccharides SIII and SVIII (47), and the cross-reaction between horse antibody globulin and normal globulin with the rabbit antiserum to the former (9)) with the result that cross-absorption did not resolve the heterogeneous antibody system into fractions of specific reactivities. A random distribution of groupings capable of reaction with heterologous antigen, accordingly has been postulated (9).

With the aid of Table IX, the cross-reactions of the following pairs of antigens with four antisera are discussed below: Native antibody (NA)—native normal globulin (NG); native normal globulin (NG)—denatured normal globulin (DG); native antibody (NA)—denatured normal globulin (DG).

*NA-NG.*—Comparison of the behavior of native normal and immune globulin toward anti-NA serum reveals that while only one-fourth of the total antibodies is precipitated by NG, the reaction constants are the same for both antigens when calculated to the same amount of total antibody,  $A$ . This finding is in qualitative and quantitative accord with the observations of Treffers, Moore, and Heidelberger (9). A similar relationship was found to exist when NA and NG were used to absorb antiserum to the denatured antibody. It may be concluded, therefore, that those rabbit antibodies to either native or denatured antibody that are precipitated by either antigen react equally with both.

However, precipitin titrations of NA and NG with antisera to native or denatured normal globulin fail to reveal an analogous similarity in antigenic structure. While

<sup>4</sup> Since DA was used as a suspension, calculations of combining ratios are meaningless in all instances where this antigen was employed. However, as was shown by Treffers and Heidelberger (7) the data may be used for the estimation of  $A$ .

normal GI was a pseudoglobulin fraction whereas the pneumococcal antibody globulin is preponderantly euglobulin. It is not possible to decide this question on the basis of the present experiments. However, the question also remains whether generally euglobulin is a genuine serum component or whether it may not represent a modified pseudoglobulin produced as a result of *in vitro* purification (21).

It has been suggested that the regenerated fraction may "consist of those antibody molecules which had escaped extensive unfolding" (48). While it is true that the diffusion method is too insensitive to detect the presence of traces of partially denatured or undenatured protein in an otherwise monodisperse solution of fully denatured material, it is doubtful that the "regenerated" material may be generally identified with undenatured protein. As discussed in detail elsewhere (14, 25), the percentage of regenerated normal globulin, serum albumin, or hemoglobin is too large to escape detection by diffusion or ultracentrifugation, if the regenerated protein departs significantly from the size or shape characteristics of the fully denatured form. It is also clear that the serological properties of the regenerated antibody fraction differ significantly from those of the native protein. As discussed above (page 431) the change in combining weight ratio is in the direction of a higher mean molecular weight (at constant antibody valence) which, admittedly, might be in accord with the sedimentation characteristics of native and regenerated antibody, given in Table I. However, this may also be explained by the assumption that the heavy component of the native antibody is more susceptible to regeneration than the normal component, or, that its ability to combine with, or to be precipitated by the antigen has become impaired.

A significant product of the present investigation is the finding that the irreversibly denatured fraction is serologically active. This may be taken to mean either that the specific configuration of the protein molecule is unrelated to the latter's behavior as an antibody, or that the serological activity is associated with structural regions which are unaffected by the denaturation process. The latter interpretation, suggested by Wright and Pauling (48), may find its analogue in the behavior of certain proteolytic enzymes which have been found to remain active for considerable time after treatment with strong urea solutions (*cf.* 14). Regardless of which viewpoint is accepted, the inference is not that antibodies cannot be denatured without incurring a loss of serological activity, but that in the sequence of changes brought about by denaturation, serological activity is among the last properties to be affected.

The fact that native and denatured antibodies are antigenically more closely related to each other than to the corresponding fractions of normal globulin provides strong evidence for hypothesis that the two proteins are chemically different entities. While absorption experiments have shown the antigenic relation between antibody and normal globulin not to be as close as previously believed (6, 9), the fact remains that those groups of the protein which are

responsible for antibody activity do not contribute to the antigenic structure. Accordingly, the observed antigenic differences between denatured antibody and denatured normal globulin are not due merely to those structural regions which might have remained unaffected by denaturation. It appears probable that more fundamental characteristics are responsible for the different behavior and properties of these two globulins although an elucidation of these factors cannot be made at the present time.

Our thanks are due to Dr. Max A. Lauffer for his generous cooperation in performing the sedimentation analyses described in this paper.

#### SUMMARY

1. The influence of guanidine hydrochloride on the denaturation and regeneration of Type I antipneumococcal horse serum globulin was determined by measurements of viscosity, diffusion, and sedimentation in the ultracentrifuge. In addition, the effect of NaCNS on the antibody globulin was studied.

2. Both the irreversibly denatured and the regenerated fractions were found to be precipitable by SI. The observed changes in combining ratio have been tentatively explained in terms of (*a*) changes in the mean molecular weight, or alternatively (*b*) an increase in the number of serologically active groups upon denaturation, followed by masking of the latter upon regeneration. Discounting a specific effect of NaCNS on either fraction, the extent of specific precipitation is of the same order of magnitude for native and irreversibly denatured antibody.

3. Quantitative precipitin titrations have been performed on rabbit antisera to native and irreversibly denatured horse antibody, and normal globulin GI, respectively. No significant differences in the antigenic activity of these proteins were found. Measurements of their cross-reactivity led to the conclusion that the native and irreversibly denatured fractions of antibody globulin are antigenically more closely related to each other than to the corresponding fractions of normal globulin, and *vice versa*.

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## METABOLISM OF TISSUE CULTURES

### III. A METHOD FOR MEASURING THE PERMEABILITY OF TISSUE CELLS TO SOLUTES\*

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Two important factors concerned in the dynamics of tissue growth are (a) the availability of circulating and extracellular foodstuffs to cells (permeability and (b) the synthesis of these foodstuffs into protoplasm (metabolism). It is clear that either permeability or metabolism might be a primary factor limiting the growth rate of tissues, and it is important to know whether tumor growth is associated with any specific anomaly in tumor cell permeability. For technical reasons much less is known about the permeability of tissue cells than about their metabolic functions. The present report describes a method by which both of these factors may be quantitatively studied in cultures of normal and tumor cells with the aid of radioactive isotopes. The results obtained by this method will be presented in succeeding papers.

Although many aspects of tissue metabolism can be investigated without the use of intact tissues, as in tissue slices or cell-free extracts, permeability relations between cells and their environment present a more difficult problem. Such is the delicacy of the processes whereby substances enter the cell that any deviation from the normal state of the tissue may cast doubt on the applicability of the results obtained. An understanding of permeability processes is fundamental to our knowledge of over-all cell metabolism, yet for only one type of cell, the erythrocyte, are there direct means for measuring these processes.

The erythrocyte is by no means an ideal cell for studying cell permeability in general, but it has the practical advantage of being accessible in large quantities and easily separable from its extracellular environment. Even with erythrocyte isotope techniques have been of great value, since they alone permit a distinction between static and dynamic equilibria and the measurement of rates (1). With isotope methods the rate of movement of substances across a barrier, as well as of metabolic turnover, can be measured without altering concentrations of the substances being studied or other environmental conditions.

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By tissue culture methods, tissue cells can be obtained in a relatively intact state for observation and analysis. In previous work, it has been possible to calculate permeability of normal and tumor cells to water by measuring the rate of their endosmotic swelling (2). The present method is based on the roller bottle method of culture (3); its adaptation to measurement of radioactivity of the cells in a culture, independently of that in the culture medium, makes possible continuous chemical measurements of the rate of passage of an isotope between medium and cells. Permeability of cells to potassium and phosphate ions has been successfully studied by this method.

### *Technique*

Tissues are cultivated in roller bottles bearing a window on one side to which a thin coverslip is attached. The inner side of the coverslip is moistened with chicken plasma (0.02 to 0.05 ml.) and 10 to 100 mg. of minced tissue is distributed over it. The wet tissue is weighed rapidly to the nearest milligram in a small covered Petri dish. After coagulation of the very thin plasma layer, 4 ml. of a medium not containing the isotope are introduced into the bottle, and rotation is begun. The control period of incubation without radioactive medium (preincubation period) lasts at least 4 to 18 hours to allow the repair of traumatized tissue and the release of intracellular material into the medium as a result of cutting cells. We have found that 30 to 50 per cent of the phosphate originally present in tissue is lost in the process of mincing and explantation; an additional small loss of phosphate takes place within the next hour or two (4).

At the end of the preincubation period, the medium is drawn off as completely as possible and radioactive medium (4 ml.) is substituted. The new medium is made up exactly like the original medium except for the addition of the radioactive substance (potassium or phosphate) in place of the corresponding non-radioactive component, and it is warmed to the temperature of the incubating culture before substitution. It is introduced into the bottle in such a way that it does not come in contact with cells. The bottle is then rotated slowly once or twice to achieve the usual distribution of medium and cells, and the initial radioactivity reading is made. Thus, the small amount of radioactive medium held in the angle where the coverslip is joined to the bottle is included in this control radioactivity reading ( $C_0$ ) before penetration of the tissue has occurred. The bottle is kept rotating about its axis of symmetry, and readings are made at intervals by means of a Geiger-Müller counter in the incubator. At the conclusion of an experiment the medium is withdrawn, tissue is washed gently with saline solution, and removed for analysis. Total phosphorus (or potassium) is determined in cells and media and radioactivity measurements are made on both.

Radioactivity readings are made with the bottle in the position shown in Fig. 1. In addition to the radioactivity of the cells, a contribution is made to the total reading by the radioactivity of the medium. This contribution is reduced by the position of the bottle to 5 to 10 per cent of the value the same amount of  $P^{32}$  would give in the position of the tissue. This is due to the

facts that the beta particles responsible for all or nearly all of the radiation from the isotopes used (1) have a much more restricted solid angle with respect to the counter than those emanating from the tissue, (2) are partially absorbed in the medium, and (3) have to traverse a longer air path between the medium and the counter.

*Calculation of True Radioactivity of Cells.*—As the radioactive isotope is taken up by the cells of the explanted tissue, there is then a progressive increase in the apparent radioactivity of the bottle read in the position shown in Fig. 1, as the isotope is translocated to a position close to the counter (a correction of each reading being made for radioactive decay, of course). Since a concurrent drop occurs in that contribution which the medium makes to the reading, increments in the reading are not exactly equal to the radioactive

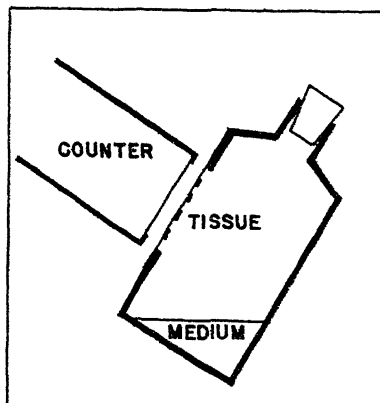


FIG. 1. Diagram illustrating relative positions of culture bottle and Geiger-Müller counter while readings of tissue radioactivity are being made.

uptake, but are proportional to it. At the end of the experiment the radioactivity of medium and tissue is determined by placing ashed samples in porcelain capsules under the counter. In this way the final radioactivity of the cells is determined under the same conditions used for determining radioactivity of media. This is called the true final cell radioactivity ( $P'_{if}$ ). Using this value, the final reading ( $R_f$ ), and the control reading ( $C_0$ ), the true cell radioactivity at any time can be calculated from the reading at that time, according to the following calculation.

The apparent radioactivity of the whole bottle at any time is made up of two components, that from the cells and that from the medium, and is the sum of the two. Let

- $R$  = apparent activity of bottle at time  $t$  (reading)
- $A$  = apparent activity of cells at time  $t$
- $C$  = apparent activity of medium at time  $t$

Then

$$R = A + C, \text{ and } R_0 = C_0 \text{ (when } A_0 = 0)$$

Since  $C$  is proportional to the amount of radioactivity remaining in the medium,

$$C = \frac{P' - P'_i}{P'} C_0$$

where  $P'$  = true total activity in bottle, and  $P'_i$  = true activity in cells. Since the apparent and true cell activities are always proportional,

$$A = \frac{A_f}{P'_{if}} P'_i$$

where  $A_f$  = observed final activity of cells (in bottle) and  $P'$  = true final activity of the cells.

Whence,

$$P'_i = K(R - C_0), \text{ where } K = \left( \frac{A_f}{P'_{if}} - \frac{C_0}{P'} \right)^{-1}$$

Since  $K$  in the last equation is constant independently of time, true cell activity is always proportional to the difference between the reading at any time and the control reading, and in the case of the final reading,

$$P'_{if} = K(R_f - C_0), \text{ or } K = \frac{P'_{if}}{R_f - C_0}$$

Therefore,

$$P'_i = \frac{P'_{if}}{R_f - C_0} (R - C_0)$$

True cell radioactivity is calculated from the readings at each time interval, and a curve of uptake of the isotope is drawn.

*Uptake of Radioactive Potassium and Phosphate.*—Curves giving the results of typical experiments on the accumulation of  $K^{42}$  and  $P^{32}$  are shown in Figs. 2a and 3a. These cultures were grown in a peptone medium which, though incomplete, allows excellent peripheral cell growth (4). The curve for  $K^{42}$  uptake approaches asymptotically a limiting value where the specific activity ( $K^{42}/K$ ) of the cells is equal to that of the medium. On the other hand, the turnover of phosphorus is far from completion even after several hours, although a rapid rate is observed at first. If one assumes that only the inorganic phosphate in the cells is capable of exchange with extracellular radioactive phosphorus, which is entirely inorganic in the medium used, it will be seen that a rapid exchange between the medium and the diffusible inorganic phosphate of the cell would result in a rapid partial turnover, followed by a slower rate of  $P^{32}$  uptake as the turnover of organic compounds caused further  $P^{32}$  exchange. On this basis, the rate of entry of  $P^{32}$  into the inorganic phos-

phate of the cells becomes of the same order of magnitude as that of  $K^{42}$  into the whole cell potassium.

Proof of these assumptions regarding the behavior of inorganic and organic phosphorus is given by the data in Fig. 4. In this experiment, a group of parallel cultures was run and individual cultures were sacrificed at intervals. The tissues were then extracted *in situ* twice during 24 hours with cold 5 per cent trichloroacetic acid. From this extract of the acid-soluble phosphorus,

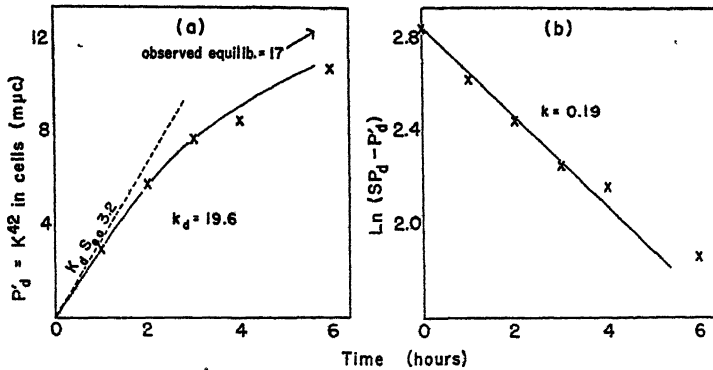


FIG. 2. Uptake of  $K^{42}$  by chick embryo muscle.

(a) Curve of observed uptake (continuous line), showing calculation of  $k_d$  from the initial slope (broken line).

(b) Logarithmic plot used in calculation of  $k$ .

The data used in calculation of these constants are the observed values of  $P'_d$ , and the following:

$$\begin{aligned} P &= 540 & S_{eo} &= 0.165 \\ P_d &= 140 & S &= 0.122 \\ & & S P_d &= 17.1 \end{aligned}$$

Derived constants:  $k_d = 19.6$ ,  $k = 0.19$ ,  $k_1 = 0.14$ .

inorganic phosphate was precipitated by an ammoniacal magnesium mixture. The specific activity ( $P^{32}/P$ ) of the cell inorganic phosphorus is seen to rise rapidly while that of the remainder of the cell phosphorus (composed of acid-soluble organic, alcohol-soluble, and "residual" P) is always much less than the inorganic phosphorus specific activity. Separate analysis of the acid-soluble organic fraction, and of the alcohol-soluble and alcohol-insoluble components of the acid-insoluble residue, shows that all of these have lower specific activities than the inorganic component. This favors the belief that the inorganic P of the medium penetrates as such and is subsequently converted into organic forms.

### Calculation of Rate Constants<sup>1</sup>

*Potassium.*—The curve for accumulation of  $K^{42}$  intracellularly at  $37^{\circ}C.$ , shown in Fig. 2, appears at first sight to indicate that equilibrium between

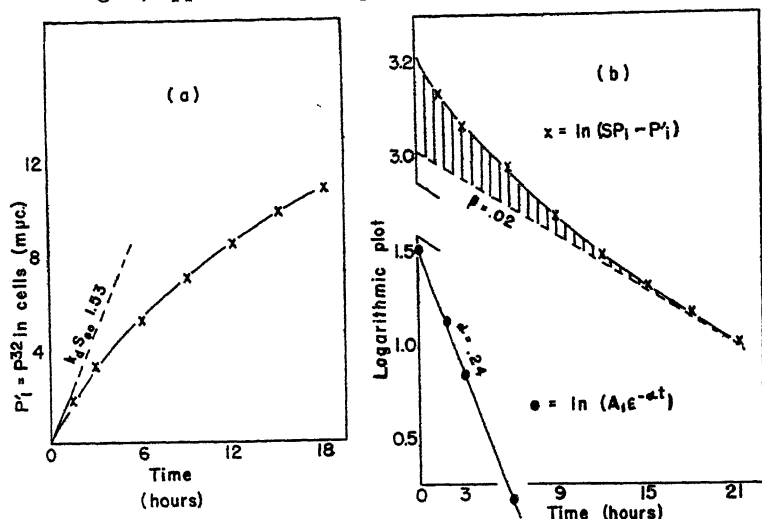


FIG. 3. Uptake of  $P^{32}$  by chick embryo muscle.

(a) Curve of observed uptake (continuous line), showing calculation of  $k_d$  from the initial slope (broken line).

(b) Logarithmic plot used in further calculations. Above, calculation of  $\beta$ ; below, calculation of  $\alpha$ . Note that the shaded area in the upper diagram is used in deriving the lower curve (see text). The  $\alpha$  and  $\beta$  lines meet the origin at  $\ln A$  and  $\ln B$ , respectively.

Data used are the observed values of  $P'_i$ , and the following:

$P_e = 50$	$S_{e0} = 0.98$
$P_d = 9$	$S = 0.48$
$P_n = 42$	$SP_i = 24.8$

Derived constants:

$A = 4.5$	$B = 20.3$
$k_d = 1.56$	$k_1 = 0.174$
$k_n = 0.50$	$k_2 = 0.012$

<sup>1</sup> In the subsequent discussion, the following symbols are used:

$t$  = time in hours.

$P$  = mass (micrograms) of the labeled atom (P or K) in the entire culture bottle.

$P_e$  = mass of P or K in the medium.

$P_i$  = mass of P or K in the cells.

$P_d$  = mass of the "diffusible" component in the cells.

$P_n$  = mass of the "indiffusible" component in the cells.

(Footnote continued on following page)

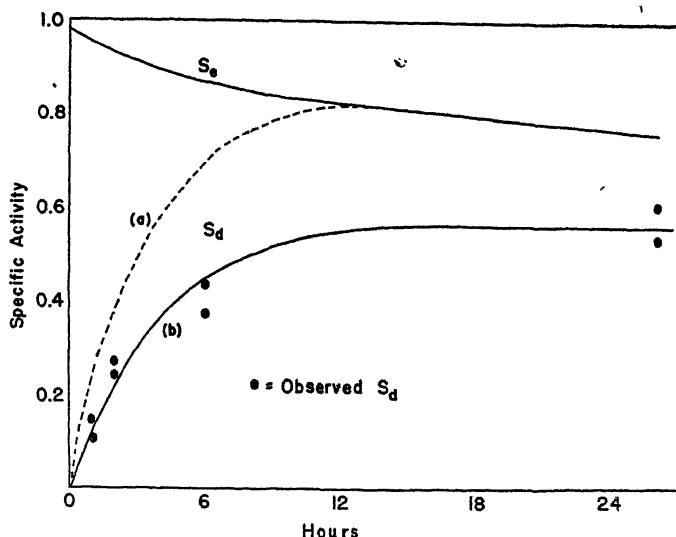


FIG. 4. Specific activity changes in cell inorganic phosphate. Upper curve: specific activity of medium ( $S_e$ ). Lower curves: calculated theoretical specific activity of the diffusible (inorganic) phosphorus of cells ( $S_d$ ); (a) corresponds to case *a* (4) in which organic phosphorus exchanges directly with phosphate of the medium, and (b) to case *b* (4) in which medium phosphate first exchanges with cell inorganic phosphate. Observed values are for cultures analyzed at 1, 2, 6, and 26 hours (two cultures in each series). The eight cultures analyzed were of comparable weight and maintained under the same conditions as the culture shown in Fig. 3. The theoretical curves are derived from the constants given there.

Note: It can be shown from equations (5) that  $S_d$  should be constant and maximal when  $(S_e - S_d)/(S_d - S_n) = k_n/k_d$ . In the case illustrated, this is approximately true between 15 and 25 hours.

(In the case of potassium,  $P_i$  and  $P_d$  are assumed identical; in the case of phosphorus  $P_d$  is assumed to be the cell inorganic phosphate.)

Then,

$$P = P_e + P_i = P_e + P_d + P_n$$

$P'$  = amount of radioactive isotope (millimicrocuries) in the culture bottle.

$P'_e, P'_i, P'_d, P'_n$  correspond to fractions with the same subscripts above.

$$S = \text{specific activity of entire culture} = \frac{P'}{P}$$

$S_e, S_i, S_d, S_n$  are specific activities of parts of the culture; e. g.,  $\frac{P'_e}{P_e}$  etc.

Further subscripts indicate the time of the observation; e. g.,

$S_{e0}$  = specific activity of medium as placed on the culture.

$S_{ef}$  = specific activity of medium when removed from the culture.



intra- and extracellular potassium  $P_e \rightleftharpoons P_d$  is a monomolecular exchange uncomplicated by any competing or following reactions. Then if  $k_d$  represents the rate of passage of potassium into the cell in exchange for potassium coming out,

$$k_d = \frac{dP}{dt} \text{ (}\mu\text{g./hour)} \quad (1)$$

and the accumulation of  $K^{42}$  (designated as  $P'$ ) intracellularly should be given by the expression:

$$\frac{dP'_d}{dt} = k_d (S_e - S_d) \quad (2)$$

$S_e$  and  $S_d$  being the specific activities of the external and internal (diffusible) phases. Upon integration, this becomes:

$$S_d = \frac{P'_d}{P_d} = S(1 - e^{-kt}), \text{ where } k = \frac{k_d S_{e0}}{P_d S} \quad (3)$$

Thus the initial rate is determined by  $S_{e0}$ , the initial specific activity of the medium, and the final equilibrium by  $S$ , that of the entire system. It is  $S_d/S$  which approaches 1 exponentially and not  $S_d/S_e$ , although the latter is customarily termed the relative specific activity.

It will be seen that  $k_d$  depends on the size of the explant; to make results on cultures of different sizes comparable, it is necessary to use  $k_1 = k_d/P_d$ . Since  $S_{e0}$  and  $S$  are related by the expression:

$$\frac{S}{S_{e0}} = \frac{P_e}{P_e + P_d}, \text{ and } k_1 = k \frac{P_e}{P_e + P_d},$$

$k_d$  is now equal to the "turnover rate" as customarily defined, and  $k_1$  is the reciprocal of the "turnover time" (5) in hours, being the proportion of the intracellular potassium exchanged per hour.<sup>2</sup>

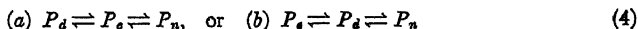
An approximate estimate of  $k_d$  can be made by drawing a line tangent to the uptake curve at its origin as shown in Fig. 2a. According to equation (2) above, the slope of this line will be  $k_d \cdot S_{e0}$  at  $t = 0$ . The constants can better be derived, using the entire series of observations, by plotting  $\log_e (SP_d - P'_d)$  against time. If the assumption were correct that the exchange is mono-

<sup>2</sup> It will be noted that the turnover rate is defined in terms of an absolute amount of potassium, and the turnover time in terms of an amount relative to that present in the cells. At the turnover time, the cell specific activity would, with infinite medium, be equal to  $1 - 1/e$  times its final value. Under the conditions stated above, this value ( $S_d = 0.693S$ ) would be reached at a time equal to the turnover time multiplied by  $\frac{P_e}{P_e + P_d}$ .

molecular and if the tissue potassium content ( $P_a$ ) remains constant, the (negative) slope should be a straight line equal to  $k$  in equation (3) above, from which  $k_a$  and  $k_1$  may be derived. This derivation of the constants from the data is shown in Fig. 2b.

As will be shown in a future paper, potassium penetration at low temperatures, and also probably at 37°, is actually a more complicated process than the one described here.

**Phosphate.**—The case of phosphate penetration is complicated by additional reactions, since phosphate is converted into organic forms existing in the cells. This is illustrated by a plot of  $\log_e(SP_i - P'_i)$ , (Fig. 3b). This line shows a steep portion during the first few hours, which is thought to represent the period during which penetration of phosphate into the cell inorganic phosphate occurs ( $P_e \rightleftharpoons P_a$ ). The later decrease in slope then represents metabolic turnover of organic phosphorus within the cell, as this reaction predominates. Since this secondary slope is approximately a straight line in the region with which we are concerned, and since we are primarily interested in the first process (permeability), we have made the simplifying assumption that the much slower metabolic turnover may be expressed by a single rate constant, although it must involve several reactions. We may now assume either (a) that cell organic phosphorus is in equilibrium directly with phosphate in the medium, or (b) that it is in equilibrium only with the small amount of intracellular inorganic phosphate; *i.e.*,



In the first case the calculations would be somewhat simpler, since the final reaction would be a summation of two reactions similar to that discussed above. The second case (b) seems more likely on *a priori* grounds, and this is borne out by the curve for cell inorganic phosphate specific activity, which as will be shown fails to rise promptly to meet that of the medium. If, as suggested by Sacks and Altshuler (6), phosphorus enters the cell through phosphorylation reactions at the cell surface; our data indicate that the rate of this process will still be best described by case (b). The following calculation is based on the equilibrium (b) above, assuming that the net amount of phosphorus in each of the three compartments is unchanged.

If we consider the rate of passage of phosphorus in either direction between medium and inorganic cell phosphate in micrograms per hour as  $k_a = \frac{dP_e}{dt}$ , and the rate of exchange between cell inorganic and organic phases as  $k_n = \frac{dP_n}{dt}$ ,  $k_a$  and  $k_n$  are the turnover rates for permeability and metabolic exchange, respectively. As before,  $k_1 = k_a/P_a$  is the proportion of the intracellular phosphate turned over (with respect to the external phase) per hour, or the recipro-

cal of its turnover time;  $k_2 = k_n/P_n$  is the proportion of the cell organic phosphorus turned over per hour, or the reciprocal of its turnover time. The movement of  $P^{32}$  through the phases can be expressed thus, as in (2):

$$\frac{dP'_e}{dt} = -k_d \left( \frac{P'_e}{P_e} - \frac{P'_d}{P_d} \right), \quad \frac{dP'_n}{dt} = k_n \left( \frac{P'_d}{P_d} - \frac{P'_n}{P_n} \right) \quad (5)$$

where  $P'_e + P'_d + P'_n = P' = \text{constant}$ , and  $P_e, P_d, P_n$  are constant. These simultaneous equations have the following solution:<sup>3</sup>

$$\begin{aligned} P'_e &= A_1 e^{-\alpha t} + B_1 e^{-\beta t} + SP_e \quad (SP_e = P'_e(t=\infty), \text{ etc.}) \\ P'_d &= A_2 e^{-\alpha t} + B_2 e^{-\beta t} + SP_d \\ P'_n &= A_3 e^{-\alpha t} + B_3 e^{-\beta t} + SP_n, \text{ with} \end{aligned} \quad (6)$$

$$A_1 + A_2 + A_3 = 0, \text{ and } B_1 + B_2 + B_3 = 0, \text{ and (considered at } t = 0)$$

$$A_1 + B_1 = P'_{e0} - SP_e = SP_i, \quad A_2 + B_2 + SP_d = 0, \text{ and } A_3 + B_3 + SP_n = 0$$

Differentiating (6),

$$\frac{dP'_e}{dt} = \frac{dP'_i}{dt} = \alpha A_1 e^{-\alpha t} + \beta B_1 e^{-\beta t},$$

and the initial slope

$$\frac{dP'_i}{dt} (t=0) = \alpha A_1 + \beta B_1 = (\text{from 5}) k_d S_{e0} \quad (7)$$

The constants in (6) now have the following relationships to those in (5):

$$\alpha + \beta = k_d \left( \frac{1}{P_e} + \frac{1}{P_d} \right) + k_n \left( \frac{1}{P_d} + \frac{1}{P_n} \right) \quad (8)$$

$$\alpha\beta = k_d k_n \frac{P_e + P_d + P_n}{P_e P_d P_n} \quad (9)$$

$$A_1 = \frac{k_d S_{e0} - \beta SP_i}{\alpha - \beta}, \quad A_2 = P_d A_1 \left( \frac{1}{P_e} - \frac{\alpha}{k_d} \right)^*, \quad A_3 = -A_1 - A_2 \quad (10)$$

$$B_1 = SP_i - A_1, \quad B_2 = -SP_d - A_2, \quad B_3 = -B_1 - B_2$$

\* This is derived from the first equation in (5).

It is now possible to derive values for  $k_d$  and  $k_n$  from experimental data showing uptake of  $P^{32}$  by a culture by graphical analysis. This is done by plotting  $\ln(SP_i - P'_i)$  against time (Fig. 3b); this is equal to  $\ln(P'_e - SP_e)$  and (from 6) to  $\ln(A_1 e^{-\alpha t} + B_1 e^{-\beta t})$ . If  $\alpha > \beta$ , this will become, after a certain time,  $\ln(B_1 e^{-\beta t})$ . A line is therefore drawn along the latter straight part of this curve; its slope will be equal to  $-\beta$  and the extrapolated line will intercept the ordinate at  $t = 0$  to give  $\ln B_1$ .  $B_1 e^{-\beta t}$  is then calculated for various time

<sup>3</sup> We are indebted to Professor Philip Franklin of the Department of Mathematics, Massachusetts Institute of Technology, for deriving this solution.

intervals,  $A_1 e^{-\alpha t}$  is obtained by subtraction, and  $\alpha$  and  $A_1$  are calculated in similar manner by plotting  $\ln (A_1 e^{-\alpha t})$  against time.  $k_d$  is then obtained from the equation  $k_d = \frac{\alpha A_1 + \beta B_1}{S_{e0}}$  and  $k_n$  from equations (8) and (9). The remaining constants are obtained by substitution into equations (10) and, finally, curves are drawn according to equations (6), which should correspond to actual uptake observations.

In making these calculations,  $P_d$  is assumed to correspond to the cell inorganic phosphate obtained from analyses of tissue, and  $P_n$  to the organic phosphorus.

An approximation method may also be used, which has the advantage of simplicity. The permeability constant can be approximated by drawing a line tangent to the uptake curve as was done in the case of potassium. This is shown in Fig. 3*a*. Using the first part of equation (5) above, this slope again is  $k_d \cdot S_{e0}$  at  $t = 0$ , and it is clear that derivation of  $k_d$  in this way is valid regardless of the complexity of further reactions involving the isotope. The initial slope is always maximal at this time, since  $S_e - S_d$  can only decrease as penetration occurs. A value for  $k_n$  can be obtained by assuming an approximate one and calculating the uptake curve ( $P'_d$ ) from the above equations.

The appropriateness of the calculation has been checked by analyses of cultures sacrificed at intervals to obtain the specific activities of phosphorus in medium and in cell inorganic and organic phases. One series of analyses is shown in Fig. 4 together with theoretical values according to cases *a* and *b* (4). It will be seen that the cell inorganic specific activity  $S_d$  never rises to meet the external  $S_e$ , as would occur in case *a* (4) if the phosphorus of the medium were exchanging directly with the organic phosphorus of the cell as well as with its inorganic phase. The same tendency of the cell inorganic specific activity to reach an intermediate specific activity has been seen in two parallel experiments of eight cultures each, using chick embryo muscle and mouse sarcoma.

The examples given in Figs. 2 and 3 have been chosen to illustrate the techniques which have been used in deriving constants from data on the uptake of isotopes by tissues. In these examples,  $k_1$  is for potassium 0.14 and for phosphate 0.174, indicating that the turnover times are about 7 and 6 hours, respectively.  $k_2$  for phosphorus is 0.012. Approximately 60 additional cultures of chick embryo muscle cultivated under the same conditions have yielded data showing that these constants are representative of this tissue in culture. In future papers we shall present these data, together with parallel data on other normal and malignant tissues grown under various conditions.

The part played by cell division in the uptake of ions will be considered in a later paper. Preliminary experiments have indicated that the permeability rates ( $k_1$ ) are little affected by complete inhibition of mitosis.  $k_2$ , on the other

hand, is greatly influenced by growth, as might be expected from our data on nucleic acid phosphorus turnover in resting and growing liver (7).

*Permeability Rates.*—Although metabolic turnover is customarily expressed by rate constants such as the above (*i.e.*, indicating the rate by which turnover approaches completion) it is usual to express permeability differently. The term permeability implies the passage of material across a surface or membrane; its rate should therefore be proportional to the area of the surface. The fact that ions must cross the cell surface in order to enter the cell does not, of course, mean that this surface is the limiting factor determining the rate of exchange between medium and protoplasm. It is desirable, nevertheless, to express the data in terms of cell surface.

An approximate calculation of total cell surface in a culture can be made on the basis of individual cell measurements. A large series of such measurements were made on spindle-shaped chick embryo cells in a previous study (8). Cells varying between 400 and 2000  $\mu^3$  in volume had ratios of surface to volume ( $\mu^2:\mu^3$ ) of 0.5 to 0.85. Spherical cells of the same volume range, which might occur in denser areas, would have ratios from 0.39 to 0.65. The surface:volume ratio of such a culture might therefore be expected to fall between 0.3 and 1.0  $\mu^{-1}$ , if all cell surfaces are intact. Translating the ratios to  $\text{cm}^2:\text{mm}^3$ , we have taken the approximate surface area of cells to be 6.0  $\text{cm}^2$  per mg. wet weight of the tissue, recognizing a possible error in either direction by a factor of 2. Final wet weights of the cultures have been calculated from the phosphorus content of the tissue, which as has been shown previously is a reasonably constant measure of tissue weight (4).

Permeability can be calculated from  $k_d$  (micrograms passing into the cell per hour) by dividing this by the estimated cell surface in  $\text{cm}^2$ , this in turn being derived from the cell phosphorus content or by direct weighing of the tissue at the end of the experiment. Converting micrograms to millimoles, permeability then appears in customary terms; *e.g.*, millimoles/ $\text{cm}^2$ /hour.

In the examples given, the permeability to phosphorus of a 31 mg. culture, where  $k_d$  was 1.56, is  $p = 1.57 \times 10^{-7}$ ; that to potassium of a 36 mg. culture with  $k_d = 19.6$ , is  $p = 14.0 \times 10^{-7}$ . The latter figure may be compared with the permeability of erythrocytes to potassium, which for various species ranges between 0.5 and  $5 \times 10^{-7}$  millimoles/ $\text{cm}^2$ /hour (9). As stated above, these figures are probably representative for this tissue.

It should be emphasized that in both cases, notably that of potassium, measurements of turnover involve two processes, either of which may be of major importance in determining the passage rate of ions across the cell surface, and their exchange with the protoplasm, where they are held in higher concentration than in the external medium. Thus, although permeabilities have been expressed in terms of cell surface the rate of permeation of the radioactive isotopes may rather be limited by the binding of these ions within the

cell than by the passage of ions into the cell. This matter will be discussed further in future papers.

#### SUMMARY

By using radioactive isotopes in tissue cultures, the rate of permeation of substances into cells can be measured independently of concurrent metabolic reactions of these substances. Techniques of obtaining and analyzing data are described. Examples are given using radioactive potassium and phosphorus.

Using cultures of chick embryo muscle, turnover time for cell potassium is 6 hours, and for cell inorganic phosphate is 7 hours in the examples cited. Permeability rates, based on estimates of the cell surface involved and expressed as millimoles per  $\text{cm}^2$  per hour, are of the order of magnitude of  $10^{-6}$  for potassium and of  $10^{-7}$  for phosphate.

We wish to thank Professors E. B. Wilson and K. S. Cole for valuable comments, and Professor Philip Franklin for deriving the method of mathematical analysis used in the case of  $\text{P}^{32}$  uptake.

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# THE NATURE AND CONTROL OF REACTIONS IN BIOLUMINESCENCE

WITH SPECIAL REFERENCE TO THE MECHANISM OF REVERSIBLE AND  
IRREVERSIBLE INHIBITIONS BY HYDROGEN AND HYDROXYL IONS,  
TEMPERATURE, PRESSURE, ALCOHOL, URETHANE, AND  
SULFANILAMIDE IN BACTERIA

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## I. INTRODUCTION

The purpose of this paper is to present a general theoretical discussion, together with fairly extensive new data from experiments, concerning the action of various factors which influence the activity of enzymes in living systems. Although numerous studies have been published dealing with various aspects of the subject under discussion, certain circumstances have provided a means of reaching a somewhat clearer and perhaps more comprehensive view than has been possible heretofore. The most important of these circumstances are, briefly, as follows:—

In the first place, the concept of a reversible denaturation of the protein catalyst has furnished a partial explanation of some familiar characteristics of the relation between temperature and observed reaction rates in biological systems; *e.g.*, the reversible decrease in apparent temperature coefficient of a given process, as the temperature is increased towards the optimum. Qualitatively, the reversible denaturation of proteins as a possible controlling factor in biological reactions has been recognized for some time by various investigators, especially protein chemists (Kunitz and Northrop, 1934; Anson and Mirsky, 1934 *a*, *b*; Mirsky, 1936; *cf.* also, Anson, in Schmidt, 1944, p. 407). Kunitz and Northrop found that crystalline trypsin could be reversibly denatured by raising the temperature or increasing the alkalinity. If the conditions favoring reversible denaturation were made extreme or held for some time the denaturation became irreversible. They also observed a pronounced optimum for pH in enzyme activity. These results are particularly interesting in connection with the present study because of the close parallelisms shown to enzyme behavior in the living cell.

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More recent evidence has indicated that the reversible denaturation has a wider significance than in relation to the usual temperature-activity curves alone. For, the equilibrium between active and inactive forms of the enzyme, in the only cases thus far adequately studied, is characterized by a large volume change; as well as by the high heat and entropy of reaction typical of such complex molecules as those of proteins (Eyring and Stearns, 1939). Consequently, this equilibrium becomes indispensable in accounting for not only the temperature effect but for two other seemingly diverse types of phenomena, namely, (a) the effects of hydrostatic pressure, and (b), the action of certain drugs in relation to both temperature and hydrostatic pressure. With regard to (a), the pressure effects on the equilibrium become increasingly apparent as the value of the equilibrium constant becomes larger, with rise in temperature. Under conditions where this constant is large, and the effects of pressure on other reactions are relatively small, pressures of a few hundred atmospheres may increase the over-all rate several-fold, by shifting the equilibrium in favor of the active form of the enzyme.

As for (b), since catalytically active and inactive forms of the enzyme exist in equilibrium, at least two types of reversible combinations of different drugs with a given enzyme may take place: (I) at bonds not involved in the denaturation equilibrium, and (II) at bonds made available through this equilibrium. Different effects of temperature and pressure on the inhibitions produced by the two respective types would be anticipated: With "Type I" the inhibition should ordinarily decrease with rise in temperature, as the enzyme-inhibitor compound is dissociated, and it should be relatively insensitive to pressure because the reaction is independent of the reversible heat denaturation of the protein. With "Type II," on the other hand, although a rise in temperature would again bring about a dissociation of the enzyme-inhibitor complex, it might at the same time increase, to an even greater extent, the bonds available for combination between the two molecules, and the net effect would thus be an increase in inhibition. Moreover, because of the large volume change involved in the denaturation equilibrium, the action of drugs which combine in a manner that is dependent upon the value of this equilibrium will generally be influenced by hydrostatic pressure. Some quantitative evidence and kinetic formulations apropos of the denaturation equilibrium in controlling the response of the luminescence system to temperature, pressure, and drugs have been set forth in recent studies (Johnson, Brown, and Marsland, 1942 *a, b*; Brown, Johnson, and Marsland, 1942; Eyring and Magee, 1942; Johnson, Eyring, and Williams, 1942; Johnson, Eyring, and Kearns, 1943; Johnson and Schneyer, 1944). The present paper amplifies and extends the previous work, by taking into account the irreversible as well as reversible inhibition of the enzyme, and also by including in the same general formulation additional factors, such as hydrogen ion concentration.

In the second place, the theory of absolute reaction rates (Eyring, 1935; Evans and Polanyi, 1935; Glasstone, Laidler, and Eyring, 1941) has provided a precise, modern physical-chemical basis for the understanding of rate processes similar to that available for equilibria. Thus, the theoretical significance of temperature, as well as the fundamental relation of hydrostatic pressure, to reactions in general have finally been clearly elucidated. An application of this theory to quantitative studies of biological processes has been made with some success, as in the investigations by the senior authors and their collaborators, referred to above (*cf.* also, Brown and Marsland, 1942; McElroy, 1943).

In the third place, the picture arrived at in this study has been greatly facilitated by particularly favorable experimental material. Luminescence furnishes a unique, natural indicator of its own reaction velocity, making it possible to record with both ease and accuracy the velocity at any instant. For, there is abundant evidence that under given conditions the intensity of the light provides a reliable index to the rate of the luminescent oxidation, and this circumstance makes possible the obtaining of accurate data during rapidly changing states of the system. Furthermore, because of the speed of observations, extensive experiments can be carried out with portions of the same suspension of bacterial cells, thus permitting unusually accurate comparisons of the effects of different factors, such as the relation of concentration of an inhibitor to both temperature and pressure. The investigations which constitute the background of our present study of bioluminescence, and which justify the more or less generally accepted assumptions on which certain aspects of the analysis are predicated, have been comprehensively treated in Harvey's recent monograph, *Living light* (1940) and review papers (Harvey, 1935; 1941). Subsequent investigations, and detailed points pertinent to the discussion, will be referred to later on.

It would hardly be possible to give fully adequate reference to the literature concerning the physical-chemical analysis of biological reactions, nor would an effort towards such an end be appropriate here. Certain modern studies, however, including some reviews and monographs, which for one reason or another have become more or less outstanding, might well be cited, without reference to whether or not we are fully in accord therewith. Among them are the following: Bělehrádek, 1935; Cattell, 1936; Clark, 1937; Crozier, 1924 *a, b*, 1926, and subsequent papers; Fisher and Öhnell, 1940; Henderson, 1930; Hoagland, 1936; Lineweaver and Burke, 1934; Meyer, 1927; Overton, 1901; Warburg, 1914, 1927; Warburg and Negelein, 1921, 1928; Warburg and Wiesel, 1912; Winterstein, 1926. Some quite recent studies in related fields appear to be in substantial accord with the ideas and treatment we have set forth (Winzler, 1943; Quastel, 1943; Goldstein, 1944).

## II. THE BIOLUMINESCENT REACTION

(a) *In Extracts*

The more recent investigations on *Cypridina* extracts by Harvey and his associates (Harvey, 1941) have provided much suggestive evidence concerning the nature of the luminescent system and the manner in which it operates. In particular, the studies of Anderson (1933, 1935, 1936, 1937), Chase (1940, 1943), Chakravorty and Ballentine (1941), Johnson and Eyring (1944), and McElroy and Ballentine (1944), have furnished cogent information. As a result it now seems possible, through a critical examination of available data, to arrive at a more detailed picture of the mechanism of the reactions involved, although the chemical identity of the molecules remains to be established. The data on the effects of hydrogen ions in bacterial luminescence, presented in this paper, lend support to the existence of reactions which appear most probable on the basis of the previous evidence. Since a clear, and in so far as possible complete, view of the reactions influencing the production of luminescence is of obvious advantage in the analysis of the rate-controlling factors, the essential points in our present understanding are briefly set forth in the following paragraphs.

In accordance with a customary notation we will designate the comparatively heat-stable, dialyzable substrate, luciferin, as  $LH_2$ , and the heat-labile, non-dialyzable enzyme, luciferase, as A. The luciferin from *Cypridina* has been greatly purified and concentrated. In the reduced state and in the absence of oxygen it may be kept indefinitely at room temperature. When oxygen, or certain other agents such as ferricyanide, are added, it undergoes an oxidation that is reversible by hydrosulfite, and is not accompanied by the production of visible light. The products do not remain long in a reversibly oxidized state, however, and fairly rapidly undergo a change leading to decomposition. The state of the luciferin may be determined simply by the addition of luciferase plus oxygen, in the presence of which reduced luciferin undergoes a luminescent oxidation while the reversibly oxidized, or decomposition products do not. In the luminescent oxidation, the total light emitted under given conditions is proportional to the amount of reduced luciferin supplied, and the intensity of the light at any moment is proportional to the product of the concentration of the reduced luciferin times that of the luciferase.

The behavior of luciferin towards oxidation shows that we must recognize at least three states of this substance: first, reduced luciferin, already referred to as  $LH_2$ , second, reversibly oxidized luciferin, which we will designate as L, and third, irreversibly oxidized, or in some manner destroyed, luciferin, which we will call  $L_1$ . In addition, from the fact that luciferin has been observed to luminesce under conditions which completely denature the enzyme, *viz.* in 95 per cent ethyl alcohol at 70°C. (Harvey, 1928) it is evidently necessary

to recognize an excited state of the luciferin.<sup>1</sup> Except under the aforementioned conditions, visible luminescence occurs only when  $\text{LH}_2$  undergoes oxidation in the presence of both molecular oxygen and luciferase.<sup>2</sup> On the basis of spectrographic and certain other lines of evidence, as set forth below, it seems safe to conclude that in both cases the radiating molecule is the luciferin,<sup>3</sup> and since excitation occurs during oxidation we may designate the excited molecule as  $\text{L}^*$ .

Further evidence that luciferin is the molecule that radiates in the bioluminescent oxidation derives from spectrographic studies with highly purified extracts (Chase, 1943). Concentrated solutions of reduced luciferin show an absorption maximum at 4350 Ångstrom units. The amount of luminescence which can be obtained on addition of luciferase plus oxygen is directly proportional to this absorption. On these grounds, the peak at 4350 Å is to be identified with  $\text{LH}_2$ . When the  $\text{LH}_2$  solution is exposed to air, and the spectrograph quickly recorded, the peak at 4350 Å is seen to shift to 4650, and then gradually disappear. The maximum intensity of luminescence, at 4750 Å, is practically identical with the luciferin absorption peak at 4650 Å (Eymers and van Schouwenburg, 1937). This evidence suggests that the substance absorbing at 4650 may be identified with L. The disappearance of absorption in this region of the spectrum would then be associated with the formation of the substance we have designated as  $\text{L}_1$ .

Two additional facts of considerable significance in the spectrographic studies of Chase are, (1) that the luciferin apparently undergoes the same oxidative reaction in purified solution without the enzyme, as in the presence of the enzyme, though at a rate only about one hundredth as fast; and (2) that there is less absorption at 4650 Å in the presence than in the absence of the luciferase. From (1) it appears that the enzyme catalyzes the same oxidative reaction, with an accompanying luminescence, that occurs during auto-oxidation, without visible luminescence. The same oxidant results in both cases. From (2), if the compound absorbing at 4650 can be identified as L, it follows that in the enzyme preparation is a catalyst, whether luciferase or other agent,

<sup>1</sup> The evidence is not sufficient to conclude whether or not this is the same excitation that occurs in bioluminescence. It is an example of the luminescence corresponding to the characteristic fluorescence or phosphorescence of molecules generally (Lewis, 1944).

<sup>2</sup> In at least one instance luminescence has been observed under conditions excluding molecular oxygen. The mechanism in this case is not clear (Harvey and Korr, 1938).

<sup>3</sup> For some time it was thought that the radiating molecule was that of the enzyme, luciferase (Harvey, 1917). More recent studies (Harvey, 1944) have shed some doubt on the validity of the evidence on which the earlier conclusion was based. We favor luciferin as the molecule that radiates, for reasons given herein.

which speeds the disappearance of L. The rapid destruction of luciferin always accompanies light emission. Under conditions where there is rapid oxidation, as with a catalyst, relatively higher concentrations of the intermediate half reduced molecules are formed. These in turn presumably oxidize and reduce each other giving a completely reduced luciferin and an oxidized excited molecule in the process. One in a few hundred of the excited molecules then emits and is not destroyed,<sup>4</sup> while most of the remaining excited molecules are destroyed. Without an enzyme speeding the oxidation the concentration of half reduced molecules is much lower, so that instead of reacting so rapidly with one another the second hydrogen is removed by oxygen without luminescence, and without destroying the luciferin. This oxidized luciferin can now be reduced and when again oxidized with a catalyst luminesces.

This explanation does not require that the oxidation without a catalyst involve the removal of different hydrogens than are removed with the catalyst. Destruction arises only because the process proceeds by a different mechanism. In this way one understands Chase's observation that the same qualitative spectral changes with and without a catalyst occur. This was unclear if one supposed different hydrogens were being removed. It remains possible, of course, that with suitable reagents and conditions other hydrogens may be removed. In fact, the hydrogens of the luciferin redox potential have too little energy to provide the energy of luminescence upon oxidation. Thus, these hydrogens, without drastic modification of the molecule, could not be the important ones in luminescence.

With regard to the decomposition of L to L<sub>1</sub>, it has been observed (Johnson and Eyring, 1944) that a concentrated preparation of luciferase (together with the other cold water extractives of powdered *Cypridina*), after being subjected to prolonged dialysis, and storage under aerobic conditions for about 2 years in the refrigerator, was capable of luminescing, without the addition of any new luciferin, simply by treating with hydrosulfite, then oxygen. These results were obtained with purified luciferase kindly supplied by Dr. A. M. Chase. One interpretation of this result, consistent with the facts already referred to, is that the L<sub>1</sub> came into equilibrium with L, an appreciable amount of which had not been removed by dialysis. When treated with hydrosulfite, reduction to LH<sub>2</sub> and subsequent oxidation by the enzyme in the presence of oxygen took place with light emission. Had the L continued to undergo irreversible decomposition, with a half life at this low temperature, of say a week, at the

<sup>4</sup> McElroy and Ballentine (1944) object to this suggestion of Johnson and Eyring (1944) that the excited luciferin molecules destroyed are those which do not luminesce, while those molecules which emit will be stabilized and can again be reduced and re-emit radiation. Their objection is that there is no evidence indicating that light emission is followed by reduction of the molecule and re-emission.

end of 2 years there would have remained  $10^{-45}$  parts; *i.e.*, none of the original supply. The more likely explanation is that as the luciferin gets dilute the chance of collision of two half reduced molecules falls rapidly so that the degradation of L to  $L_1$  is much less complete than the above first order rate calculation indicates.

The possibility of reversal of the degradation L to  $L_1$  could be determined by reducing large amounts of degraded luciferin and determining whether one gets proportionately more light upon adding hydrosulfite and oxygen. A negative result would provide compelling evidence of a falling off in the rate of degradation of L with time such as could only come from the reaction being of higher order with respect to half reduced luciferin or to the disappearance of an effective catalyst of destruction.

Through the kindness of Dr. L. Michaelis, some natural and synthetic compounds with one or more properties suggestive of a relationship to luciferin were made available to us. They included the following: glucosolloxazine, *n*-methylalloxazine,  $\alpha$ -araboflavin, 6,7-dimethyl-9, *l*-araboflavin, phenalloxanthin, phenazine,  $\alpha$ -oxyphenazine, and pyocyanine. Each of these substances was tested for a capacity to luminesce under the same conditions that luciferin was observed to luminesce (Harvey, 1928); *i.e.*, in 95 per cent ethyl alcohol at 70°C. Sodium hydrosulfite was added to the alcoholic solution at room temperature, before heating to 70°. Aeration was accomplished by vigorous shaking of the test tube. No luminescence was observed under these conditions, or after the addition of oxidizing agents, including  $\text{KMnO}_4$ ,  $\text{H}_2\text{O}_2$ ,  $\text{K}_3\text{Fe}(\text{CN})_6$ ,  $\text{Ca}(\text{OCl})_2$ , or  $\text{H}_2\text{PtCl}_6$ . Thus, although no luciferase enzyme preparation was available for testing a possible luminescent reaction with these compounds, the above results indicate that luciferin is not to be identified with any of the substances tested.

The chemical identity of luciferin remains to be established. From a consideration of spectrographic and other data the possibility has been noted (Eymers and van Schouwenburg, 1937; Doudoroff, 1938) that a flavoprotein is concerned in luminescence, and there is some evidence suggesting that luciferin might be a flavin prosthetic group of a specific enzyme (Johnson and Eyring, 1944). The absorption spectrum and emission spectrum of the *Cypridina* system, and the emission spectrum of many luminous bacteria approximate the fluorescent spectrum of riboflavin (Eymers and van Schouwenburg, 1937). These bioluminescent spectra are also closely related to the luminescent spectrum of 3-aminophthalhydrazide, which like the fluorescent alloxazine nucleus of the flavin has two amino groups adjoining carbonyl groups attached to a benzene ring. The general presence of yellow pigments in organs of luminescence, together with quantitative analytical evidence of an excessively high flavin content in the light organ of the firefly (Ball and Ramsdell, 1944) also indicate an intimate rôle of flavins in light emission. On the other hand,

in a micro analysis, Chakravorty and Ballentine (1941) failed to detect the presence of nitrogen in highly purified *Cypridina* luciferin. More recently, McElroy and Ballentine (1944) have obtained evidence that phosphate is liberated during the luminescent oxidation of purified luciferin by luciferase and associated substances in the cold water extracts of *Cypridina*. Anderson and Chase (1944) have expressed doubt that luciferin is to be identified with riboflavin, chiefly on the basis of the absorption spectra, and of the behavior of preparations containing luciferin with respect to oxidation-reduction potentials. Similar arguments have been advanced by McElroy and Ballentine. Korr (1936), Anderson (1936), and Chakravorty and Ballentine (1941) have noted the possibility that luciferin may be a polyhydroxy benzene. The study of oxidation-reduction potentials of flavoproteins by Michaelis, Schubert, and Smythe (1936) has shown that the semioxidized form comes into equilibrium in amounts of the same order of magnitude as the fully oxidized and fully reduced forms. Inasmuch as the data of Chase show no evidence of an intermediate form which might be interpreted as LH, in the oxidation of luciferin, (unless LH and LH<sub>2</sub> have the same absorption, which is not likely), the results of these two studies indicate that luciferin is not an ordinary flavin.

In the scheme of reactions set forth below, we have undertaken to systematize our knowledge and to provide a working hypothesis for interpreting the phenomena discussed in this paper. This hypothesis agrees in part with the mechanism of reactions suggested by Weiss (1938). It includes as a key intermediate, the semioxidized form of luciferin, LH.

In addition to the notation already given we will refer to the several reactions first by number, and later by either rate constants,  $k$ , or equilibrium constants,  $K$ , with subscripts to specify the reaction number. The backwards reaction is assumed to be small in comparison with the forwards reaction unless otherwise noted.

With luciferase		Additional reactions that occur with and without luciferase	
(1)	$AL + XH_2 \rightleftharpoons ALH_2 + X$	(1')	$L + XH_2 \rightleftharpoons LH_2 + X$
(2)	$A + LH_2 \rightleftharpoons ALH_2$		
(3)	$ALH_2 + O_2 \rightleftharpoons ALH + HO_2$	(3')	$LH_2 + O_2 \rightleftharpoons LH + HO_2$
(4)	$ALH \rightleftharpoons AL^- + H^+$	(4')	$LH \rightleftharpoons L^- + H^+$
(5) alpha	$AL^- + LH \rightarrow AL^* + LH^- \rightarrow AL + LH^- + h\nu$	(5') alpha	$L^- + LH \rightarrow L^* + LH^- \rightarrow L + LH^- + h\nu$
(5) beta	$AL^- + LH \rightarrow AL + LH^-$	(5') beta	$L^- + LH \rightarrow L + LH^-$
(5) gamma	$AL^- + LH \rightarrow AL_1 + LH^-$	(5') gamma	$L^- + LH \rightarrow L_1$
(6)	$ALH + O_2 \rightarrow AL + HO_2$	(6')	$LH + O_2 \rightarrow L + HO_2$
(7)	$AL + O_2 \rightarrow AL_1$	(7')	$L + O_2 \rightarrow L_1$

With regard to this scheme of reactions, certain points call for specific mention. The more important include the following.

In the absence of luciferase, reaction (1) takes place either prior to the extraction of the luciferin, or is intentionally brought about, whenever the pre-

parations have to some extent undergone the auto-oxidation, by adding reductants,  $XH_2$ , such as hydrosulfite. In living systems glucose is an especially suitable hydrogen donator. Ordinarily, with purified luciferin stored in a reduced state, an oxidation, presumably reaction (3), occurs on exposure to oxygen. Either with or without luciferase, in a subsequent step  $HO_2$  oxidizes LH or some other constituent. There is no evidence as to whether small amounts of  $H_2O_2$  are formed. It is interesting to note, in this connection, that the addition of catalase, as well as certain other agents known to catalyze the destruction of hydrogen peroxide, will accelerate the luminescent oxidation of aminophthalhydrazide (Drew, 1939). In cells, catalase would undoubtedly prevent the accumulation of  $H_2O_2$ , and luminous bacteria have been shown to contain abundant catalase (van Schouwenburg, 1940).

The kinetic analysis of the "flash" of luminescence in bacteria, occurring when oxygen is suddenly admitted to cells that have been partially or completely anaerobic, indicates the presence of two successive first order reactions (Chance, Harvey, Johnson, and Millikan, 1940; Schoepfle, 1940). The first and second steps in (5) alpha are the two important rates. The first step appears first order because there is a large percentage of  $AL^-$  going with negligible percentage change in LH.

Either with or without luciferase, the relative amounts of reactions (5), alpha, beta, and gamma, may be influenced by various factors, such as the type of oxidant, the pH, presence of specific ions, etc. In the absence of luciferase, the amount of reaction (5) alpha must be extremely small, since no visible light ordinarily appears. The manner in which certain factors operate to influence the course of (5) can best be illustrated with the aid of a potential energy diagram as shown in Fig. 1. The fact that more total light is produced from a given amount of purified luciferin in the presence of the chloride ion, and less total light in the presence of thiocyanate and certain other ions (Anderson, 1937), presumably occurs through influencing the relative extent of these reactions. Variations in the amount of destruction of 3-aminophthalhydrazide during luminescent oxidation are known to occur as the result of certain conditions (Drew, 1939), and presumably the same mechanism is again involved since Drew has shown that the reactions corresponding to our (5) beta may actually occur as well as (5) alpha.

The difficulty of dialyzing the luciferase completely free of the luciferin (Giese and Chase, 1940; Johnson and Eyring, 1944) indicates that the combination must be a fairly strong one, in this way resembling the combination between the flavin prosthetic group and its protein enzyme described by Theorell (1935).

The rate-determining reaction in the extracted *Cypridina* system depends upon the state of the luciferin, as Anderson has shown. When the luciferin



is all present as  $\text{LH}_2$ , as when pretreated with sufficient hydrosulfite, reaction (2) is rate-determining at the start. As the oxidation proceeds, some of the  $\text{LH}_2$  undergoes a gradual, dark auto-oxidation, and this, together with the molecules of L that have undergone light emission, may be reduced by agents present in the solutions. In competition with the reduction of L is the catalytic destruction of the L referred to in the previous paragraphs, but the net result is that during the latter part of the reaction the rate of reduction of L governs the velocity of the luminescent oxidation, and reaction (1) becomes the pace setter. Under these conditions, a long, dim afterglow may follow the more or

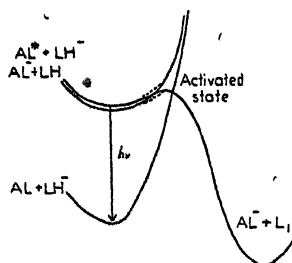


FIG. 1. Three possible energy states as ordinates are plotted against the reaction coordinate as abscissa. To the left of the activated state the distance between  $\text{AL}^*$  and  $\text{LH}^-$  or  $\text{AL}^-$  and  $\text{LH}$  or  $\text{AL}$  and  $\text{LH}^-$  respectively is the abscissas. The amount of the attractions between the respective pairs indicated by the minima on the left is not known experimentally. Passing over the activated state may involve a reaction with  $\text{O}_2$ . The middle curve corresponds to the distance between  $\text{AL}^- + \text{LH}$  decreasing until in the dotted region there is an electron transfer when the system shifts to the upper level returning to the left as  $\text{AL}^*$  and  $\text{LH}^-$ .  $\text{AL}^*$  may then radiate becoming non-excited  $\text{AL}$ . On the other hand instead of forming excited  $\text{AL}^*$  the system may pass irreversibly through the activated state or it may return unchanged as  $\text{AL}^-$  and  $\text{LH}$ .

less rapid, initial stage of the reaction. Even with highly purified preparations of luciferin, and dialyzed luciferase, a critical study of the data indicates that towards the end of the reaction the velocity constant decreases, as it would if reaction (1) replaced reaction (2) as the pace setter (*cf.* for example, the figures in Johnson and Chase, 1942). If  $\text{XH}_2$  is present in large excess, then with either reaction (1) or (2) as the dominant reaction governing the over-all velocity, it should appear to be first order with respect to the concentration of luciferin added, and a plot of log-light intensity against time should give a straight line. Furthermore, both reactions (1) and (2) are slower than the remainder under ordinary circumstances. Thus, Chance, Harvey, Johnson, and Millikan (1940) found that in crude *Cypridina* extracts, the slowest reaction is the formation of  $\text{ALH}_2$ , the luciferin-luciferase complex that reacts with oxygen.

The reactions listed above are such as to make it appear that an important

function of the luciferase in catalyzing the reaction is to ionize the LH. Evidence for the rôle of ionization will be presented in connection with the relation of bacterial luminescence to pH.

(b) *The Bioluminescent Reaction in Bacteria*

Luminous extracts have not as yet been obtained from bacteria, and anything that disrupts the cell structure appears to destroy, at the same time, capacity for luminescence (Korr, 1935). Indirect evidence, however, from similar emission spectra and other sources, indicates that fundamentally the same luminescent system operates in bacteria as in *Cypridina* extracts. In the following discussion we will assume that the same reactions, set forth above for extracts, along with some additional reactions, occur also in bacteria. The available data indicate that in a large measure such an assumption is justified.

Non-proliferating bacteria aerated in buffered NaCl at favorable temperatures emit a constant over-all luminescence for periods of some time; minutes, or sometimes hours. Reaction (5) alpha is evidently proceeding at a constant rate, and since this depends on the slower reactions (1) and (2) we must conclude that  $\text{LH}_2$  is being formed as fast as it is used up, provided there is no change in the activity of the enzymes. Reaction (1) is important in maintaining a continuous luminescence; *i.e.*, the over-all intensity is governed in part by the rate of reduction of L. This is shown by the effects of glucose on the "total light" of washed cell suspensions (Johnson, 1939), as well as by the investigations of the "flash" following oxygen deficiency, (Harvey, 1932; Johnson, van Schouwenburg, and van der Burg, 1939; Schoepfle, 1940, 1941). The "flash" has been studied in relation to the influence of substrate, time of anaerobiosis, temperature, and certain inhibitors. The evidence indicates that in the absence of oxygen, reaction (1) comes into an equilibrium depending on the amount of  $\text{XH}_2$ . The  $\text{XH}_2$  may be formed from glucose either aerobically or anaerobically. When glucose is added anaerobically, the  $\text{XH}_2$  accumulates over a period of time, progressively changing the equilibrium in favor of more  $\text{ALH}_2$ , as shown by the flash intensity when oxygen is admitted. The amount of A and of  $\text{LH}_2$  as well as  $\text{XH}_2$ , however, are important in determining the steady state value of  $\text{ALH}_2$ , and hence the over-all rate of the luminescent reaction in the presence of oxygen. The situation is obviously complicated, and it is only because some of the reactions are important or fast in comparison with others, that an analysis may be made with respect to the over-all process. By changing the conditions, different reactions may be made more important than previously in rate control. Under ordinary conditions favorable to luminescence, when there is a constant intensity, the reactions are in a steady state. With oxygen not limiting, and with an excess of appropriate substrate, glucose in particular, the amount of  $\text{XH}_2$  may be maintained essentially constant. The over-all reaction may thus be considered limited by reaction

(2), and the intensity of luminescence ( $I$ ) is then given by the following equation, in which  $b$  is a proportionality constant:

$$I = b k_2 (A) (LH_2) \quad (8)$$

With regard to the action of inhibitors, it has been shown (Johnson and Chase, 1942) that certain drugs combine reversibly in the purified luciferin plus dialyzed luciferase system of *Cypridina*, retarding the rate of the reaction without affecting the total light produced. The combination presumably takes place with the enzyme, although kinetic evidence alone is not sufficient to distinguish between enzyme and substrate as the site of such a combination. The inhibitors studied in this regard include several sulfonamides, *p*-aminobenzoic acid, and ethyl urethane. By inference, numerous other drugs may act in the same way (Taylor, 1934), although some substances appear to combine with purified luciferin, reducing the total light (cyanide; Giese and Chase, 1940), and others evidently act by quenching (azide; Chase, 1942). In bacterial luminescence, for purposes of analysis, the luciferase may be considered the chief site of the effects of pressure, temperature, and drugs such as sulfanilamide and urethane. On the whole, the analysis has been largely satisfactory, although evidence of complicating factors is also apparent.

Omitting the derivations, which have been given in earlier publications, we arrive at the following expressions with regard to the action of inhibitors that enter into an equilibrium with the enzyme, independently of the reversible denaturation (Type I), or in relation to it (Type II). The notation is as follows:  $I_1$  = intensity of luminescence before adding inhibitor;  $I_2$  after adding inhibitor; equilibrium constants,  $K$ , and thermodynamic constants,  $\Delta F^\circ$ , etc., have their usual meanings, with subscripts referring to type of inhibition, viz. "1," for the normal reversible denaturation equilibrium, "2," for the equilibrium of luciferase with Type I inhibitor, and "3," for the equilibrium involving a Type II inhibitor;  $X$  = molar concentration of Type I inhibitor;  $r$  = number of molecules of Type I inhibitor combining with each enzyme molecule;  $U$  = molar concentration Type II inhibitor;  $s$  = number of molecules of Type II inhibitor combining with each enzyme molecule;  $p$  = hydrostatic pressure.

#### Type I

$$\begin{aligned} \left( \frac{I_1}{I_2} - 1 \right) &= K_2 X^r = X^r e^{-\Delta F_2^0/RT} \\ &= X^r e^{-\Delta H_2^0/RT} e^{\Delta S_2^0/R} = X^r e^{-\Delta H_2^0/RT} e^{-p(\Delta V_2/RT)} e^{\Delta S_2^0/R} \quad (9) \end{aligned}$$

#### Type II

$$\begin{aligned} \left( \frac{I_1}{I_2} - 1 \right) \left( 1 + \frac{1}{K_1} \right) &= K_3 U^s = U^s e^{-\Delta F_3^0/RT} \\ &= U^s e^{-\Delta H_3^0/RT} e^{\Delta S_3^0/R} = U^s e^{-\Delta H_3^0/RT} e^{-p(\Delta V_3/RT)} e^{\Delta S_3^0/R} \quad (10) \end{aligned}$$

At this point it is perhaps useful to systematize, with the aid of a diagram (Fig. 2), our present information regarding the relation of the luminescent oxidation to the general respiratory pathway in bacteria in terms of the modern understanding of the mechanisms of cellular oxidations (Ball, 1944).

The scheme shown in Fig. 2 is a somewhat further specification of the one arrived at by van Schouwenburg (1938). Arrows point to the site of action of

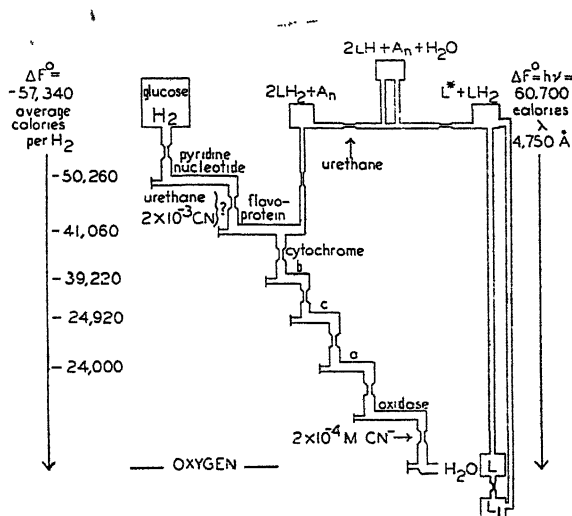


FIG. 2. Schema of the currently accepted sequence of respiration processes and on to which has been joined the luminescence process. The drawing is not to scale but we have indicated the free energy which each system liberates as two hydrogens (or two electrons + 2 protons) are liberated to form water. Constricted tubes indicate the chemical inertia of processes which may be much further inhibited by reagents written near the sensitive pathway. All the hydrogen or electron acceptors can enter into a variety of side reactions suggested by the closed off pathways to the left. The flavoprotein, in spite of its energy being lower than  $LH_2$  will serve as a hydrogen transfer catalyst to  $LH_2$  providing the hydrogens on the flavoprotein are not drawn off too rapidly by the cytochromes or by side reactions. Proportionate poisoning of oxidation and luminescence indicates action on an intermediate common to both processes. This intermediate is taken to be flavoprotein.

inhibitors, as indicated by the available data in this regard. Thus, the luminescent system shows an especial sensitivity to various narcotics, and is generally affected to a much greater extent than total oxygen consumption. Some of the narcotics have been shown to act on the extracted *Cypridina* system in comparable concentrations. On the other hand, the bacterial luminescent system is relatively insensitive to cyanide, which drastically reduces the rate of oxygen consumption, presumably by acting on the cytochrome oxidase. In high concentrations, cyanide reduces oxygen consumption and lumines-

cence proportionately, and under these conditions possibly affects a flavo-protein system. Some extracted flavoproteins may be inhibited by cyanide (Ball, 1939). The higher concentrations of narcotics like urethane, which considerably affect the rate of oxygen consumption as well as intensity of luminescence, very likely affect more than one system. However, from the presumed proximity of the luminescent system to the first steps in the dehydrogenation of the substrate, there is less opportunity for antecedent reactions to become pace setting in the process of luminescence than in the overall oxygen consumption. Under appropriate conditions, any one of the whole sequence of consecutive reactions involved in transfer of hydrogen from substrate to oxygen could become pace setting, but relatively few of these reactions precede luminescence. The measurement of light intensity, therefore, should provide a less complex, more direct approach in the living cell to the action of metabolic inhibitors which act upon those systems which influence its

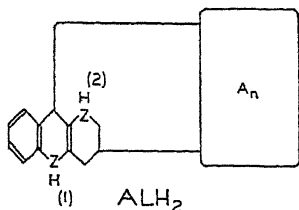


FIG. 3a

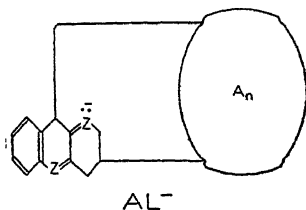


FIG. 3b

FIG. 3. (a) Hypothetical model of the luciferase molecule in the reduced state. The "Z" is for an unknown atom or group on which the oxidizable H occurs. (b) Hypothetical model of the luciferase molecule in the activated state.

activity. With regard to the actual light-emitting molecules, it will facilitate the discussion to use a tentative model, suggested both by the available data, and by the phenomena described in this paper. The model shown in Fig. 3 has obviously been influenced by Theorell's flavoprotein (Theorell, 1937), as well as by the structure of some molecules that give rise to pertinent chemiluminescences. It should be emphasized, however, that more evidence is yet needed before the structure of the prosthetic group can be specified. As indicated in Fig. 3 we assume that one hydrogen atom, number (1), is removed first to give LH, and then one proton, resulting in  $L^-$ . These assumptions are made to account for the fact that excessive concentrations of either hydrogen or hydroxyl ions interfere with the light-emitting oxidation, since  $H^+$  would oppose the formation of  $L^-$ , while  $OH^-$  would remove the proton from LH, and either effect would retard the reaction.

### III. EXPERIMENTAL METHODS

The data analyzed in this paper have been obtained exclusively in experiments with bacteria rather than extracts. Brief remarks should suffice with regard to

experimental methods, since fully detailed accounts have been published in the papers referred to earlier. Most of the experiments have been carried out with the psychrophilic, marine species, *Photobacterium phosphoreum*, which has the advantage that it produces a brilliant luminescence at room temperature. It was cultivated at 15° C. on nutrient agar containing 3 per cent NaCl, 1 per cent glycerol, and 0.5 per cent CaCO<sub>3</sub>. The cells were placed in a solution which has been referred to as "PN," made by adding equal parts of M/2 NaCl and M/4 phosphate buffer at the desired pH. A stock suspension in PN was aerated for 20 to 30 minutes, after which it usually maintained a constant luminescence intensity, during continued aeration, for another half hour, more or less. Portions of the suspension, cooled at once, following initial aeration at room temperature, to between 3 and 5°, remained constant for some hours, and on rewarming to room temperature would recover their

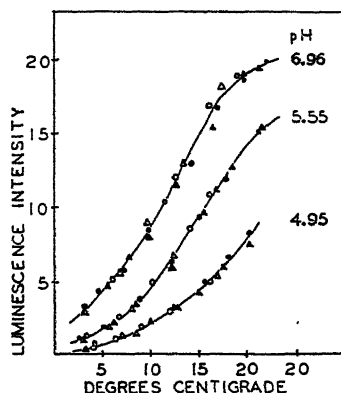


FIG. 4. Luminescence in relation to temperature in phosphate buffer and sodium chloride at three pH values. The repeated readings for each curve were obtained by cooling and warming the same suspension three successive times over the temperature range shown. The species of bacteria used for this and succeeding figures, except where specified otherwise, was *P. phosphoreum*.

former intensity. Such stock suspensions were always prepared for experiments that involved measurements of luminescence in a given portion over a period of more than 15 or 20 minutes. Actually, under conditions of temperature, etc., which do not bring about any appreciable destruction of the luminescent system, the intensity may remain constant for considerably longer periods than this. Fig. 4 shows that closely reproducible readings may be obtained with regard to the luminescence intensity at different temperatures, when portions of a given suspension, adjusted to different pH values, are warmed and cooled, over a considerable range of temperatures, three times in succession.

With the "fresh water" species, *Vibrio phosphorescens*, 0.9 per cent NaCl was used in the agar medium, and the PN was diluted with 2 volumes of distilled water. Because of the higher optimum temperature of this organism, cultures were incubated at 25° C., and the stock suspensions were maintained at about 15° C. Apart from these differences, the same procedures were followed with both species.

In most experiments, luminescence was measured by means of a photoelectric cell and d. c. amplifier, but in certain cases, such as very dim luminescence, visual photometry was employed with the aid of a modified Leeds and Northrup Macbeth illuminometer. The latter was always used for the experiments involving hydrostatic pressure.

Hydrostatic pressure at the desired temperature and other conditions was applied by means of a specially constructed bomb (Fig. 5), gold-plated to avoid contact of the bacterial suspension with physiologically active metals. The suspension was introduced through a gold-plated needle valve, and pressure applied from a hydraulic pump. Luminescence was viewed through a window of herculite, half an inch in diameter and of equal thickness. The entire bomb was held in a constant tempera-

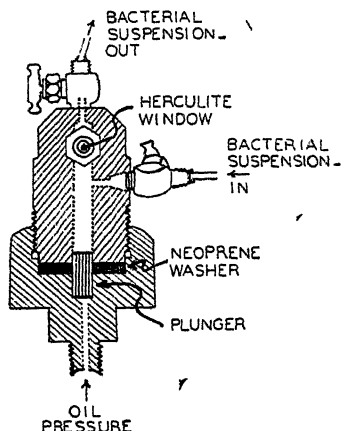


FIG. 5. Diagram of the gold-plated, high-tensile bronze pressure bomb. The bacterial suspension could be introduced and pressure applied within about 1 minute, with the bomb maintained at a given constant temperature in a water bath.

ture water bath. Temperature equilibration was rapid because of the small capacity, about 12 cc., of the bomb, and the large amount of conducting surface. Actual filling of the bomb and application of pressure could be completed within a few seconds.

#### IV. LUMINESCENCE IN RELATION TO pH

##### (a) *Influence of the Buffer System*

The relation between pH and the activity of a given enzyme system is influenced by the specific buffer system used in regulating the hydrogen ion concentration, because the equilibrium between the enzyme and the anion, as well as the cation, is significant in determining the over-all rate of catalytic action. The situation appears to be fundamentally the same as in the combination of hydrogen ions with wool, which as Steinhardt (1941) has shown varies

with the buffer system. In the luminescent system, the intensity at a given pH in some instances varies greatly with the buffer mixture (Table I).<sup>5</sup> A complete analysis of these relations would involve an extended study with the different systems. For present purposes, a full analysis with a single system is clearly more desirable.

Under most conditions phosphate appeared to be the best system for luminescence in bacteria. In approximately isotonic concentrations ( $M/4$ ; *cf.* Johnson and Harvey, 1938) phosphate reduced the intensity of luminescence at acid pH values, although little effect was noticeable at neutrality. These relations are apparent in Fig. 6, which shows the effects of various phosphate concentrations, added to 3 per cent NaCl in order to maintain the osmotic pressure. The increasing "toxicity" of increasing concentrations of phosphate at a low pH is very evident. In Fig. 6 there is also apparent a tendency for luminescence to recover with time, following the initial reduction in intensity on

TABLE I

*Influence of the Buffer System on Luminescence of P. phosphoreum at Similar pH Values. Temperature 22°C.*

	Buffer mixture								
	Phosphate			Phthalate			Acetate		
	$M/2$ NaCl $M/4$ phosphate	2 parts 2 parts		$M/2$ NaCl $M/4$ phthalate	2 parts 1 part		$M/2$ NaCl $M/4$ acetate	2 parts 1 part	
pH . . . . .	5.5	6.0	6.5	5.5	6.0	6.5	5.5	6.0	6.5
Intensity of luminescence . . . . .	25.7	28.4	28.6	12.5	26.0	26.7	<1.0	<1.0	6.9

addition of the small volume of a concentrated cell suspension to the various solutions indicated. This recovery is due in part to the decreased concentration of inhibitory metabolites, present in the thick stock suspension, that occurs when the suspension is diluted. Other factors, not yet fully worked out must also be concerned, some of which are related to temperature. Thus, at higher than optimum temperatures, the irreversible decrease of luminescence which occurs logarithmically with time takes place faster with increase in hydrogen ions (Fig. 25). At lower and lower temperatures, however, there is an increasing tendency for the logarithmically declining luminescence intensity to come to a halt, and a recovery process to set in. The latter is obviously a different reaction than the one which brings about the initial reduction in luminescence. Phenomena of slow diffusion or differential permeability of membranes to ions (*cf.* Danielli and Davson, 1943) may be involved in these relatively slow after effects.

<sup>5</sup> The assumption is made, of course, that the hydrogen ion concentration at the enzyme is the same, or proportional to, the concentration as measured in the medium.



(b) *Relation between Luminescence and pH of Phosphate-Buffered NaCl at Optimum Temperature*

From the point of view of analyzing the relation between pH and luminescence under given conditions, the most satisfactory results were obtained by adding a very small volume, generally less than 0.1 cc. of 6 N NaOH or 6 N HCl, respectively, to 10 cc. portions of an only moderately concentrated cell

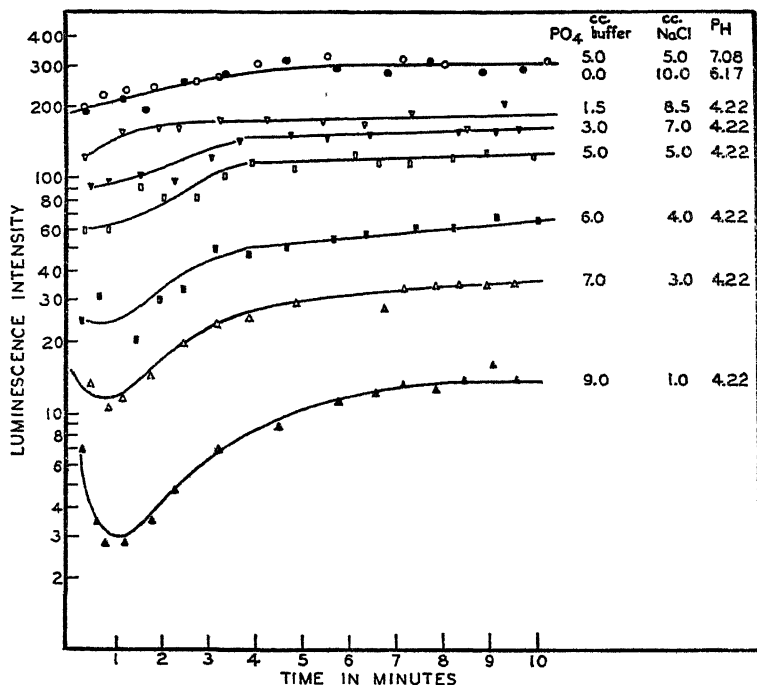


FIG. 6. The influence of phosphate concentration on luminescence intensity in approximately isotonic sodium chloride solution at various pH values. Semilogarithmic scale.

suspension in PN at an initial pH of 7.0. In this manner there was a minimum change in the suspension medium aside from the actual concentration of  $H^+$  and  $OH^-$  ions. The acid or alkali was added by serological pipette while the suspension was being rapidly stirred, with an electric stirrer, or by vigorous bubbling of air, in order to assure a quick distribution of the ions. This precaution was found necessary. Figs. 7 and 8 show that the change in intensity which takes place within the first minute following such a procedure is fairly constant with time, although there is again a tendency for recovery provided the initial effect is not too drastic.

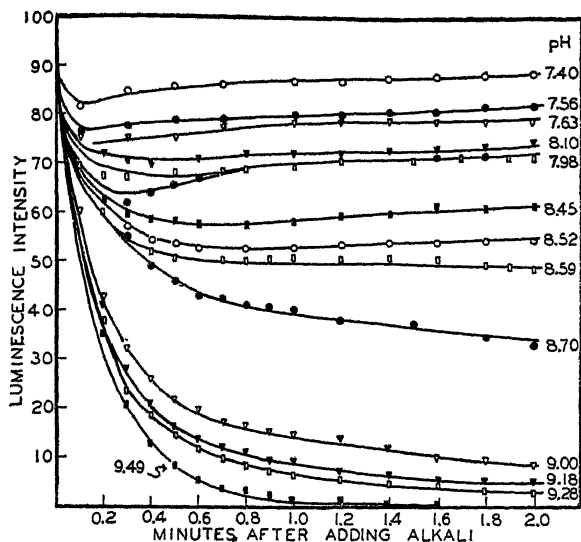


FIG. 7. The effect of changing the pH to the alkaline side of neutrality on the intensity of luminescence. Room temperature (approximately  $22^{\circ}\text{C}.$ ).

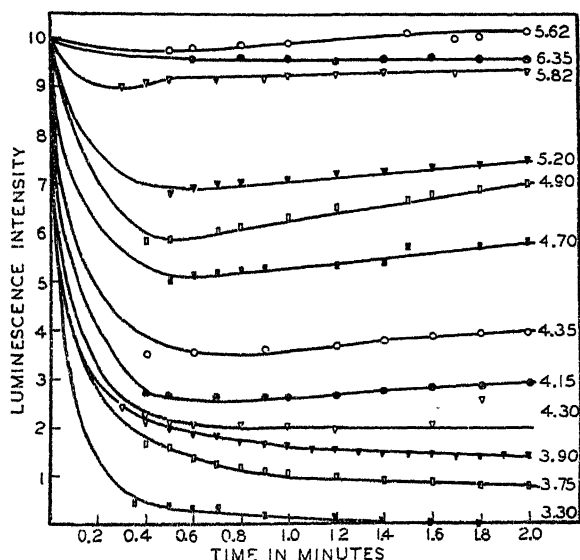


FIG. 8. The effect of changing the pH to the acid side of neutrality on the intensity of luminescence. Room temperature (approximately  $22^{\circ}\text{C}.$ ).

(b) *Relation between Luminescence and pH of Phosphate-Buffered NaCl at Optimum Temperature*

From the point of view of analyzing the relation between pH and luminescence under given conditions, the most satisfactory results were obtained by adding a very small volume, generally less than 0.1 cc. of 6 N NaOH or 6 N HCl, respectively, to 10 cc. portions of an only moderately concentrated cell

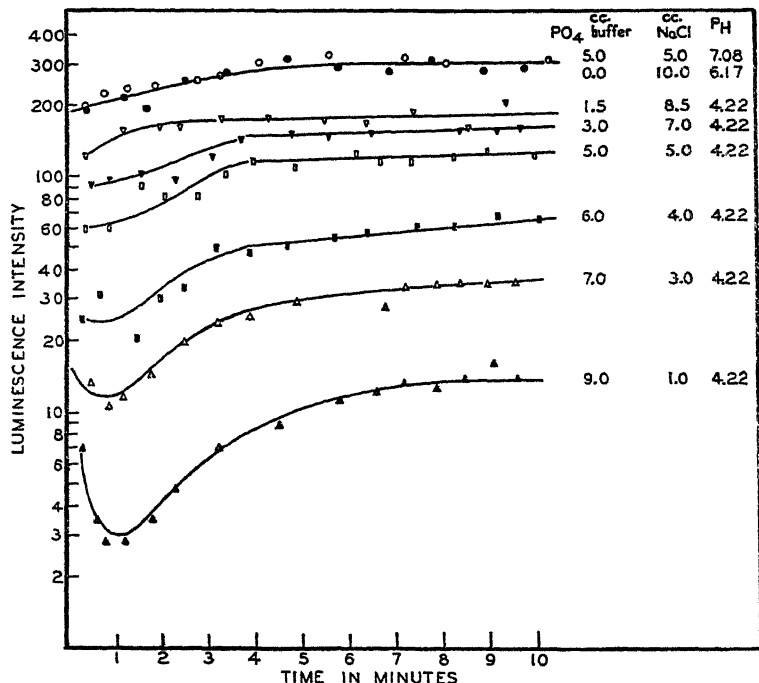


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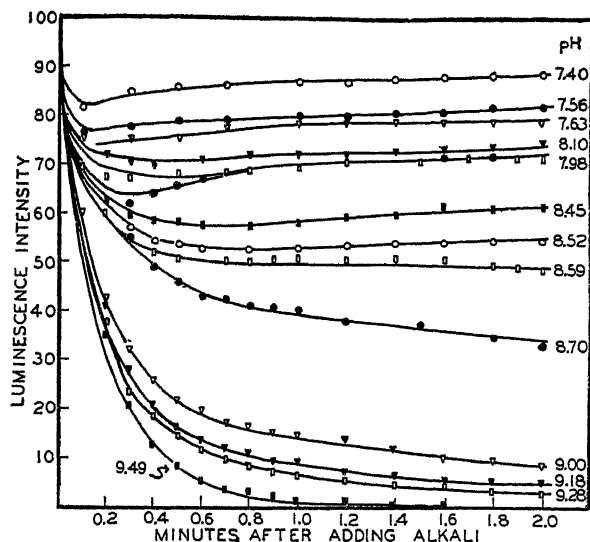


FIG. 7. The effect of changing the pH to the alkaline side of neutrality on the intensity of luminescence. Room temperature (approximately 22° C.).

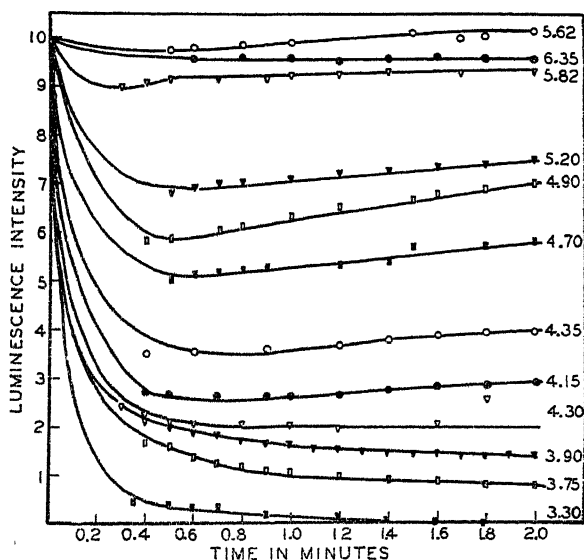


FIG. 8. The effect of changing the pH to the acid side of neutrality on the intensity of luminescence. Room temperature (approximately 22° C.).

The reversibility of the action of strong alkali is shown in Fig. 9. Similar effects were obtained when acid was first added, then neutralized. A certain amount of destruction is evident, as might be expected at the extremes of pH, but even after luminescence has decreased to about 1 or 2 per cent, the intensity quickly returns to nearly 70 per cent of its former value when the acid or alkali is neutralized. The effects are thus largely reversible, permitting analysis on the basis of equilibria. With regard to the relation between concentration and inhibition at a given temperature and hydrostatic pressure, we may take the maximum luminescence intensity as  $I_1$ , at the pH where this

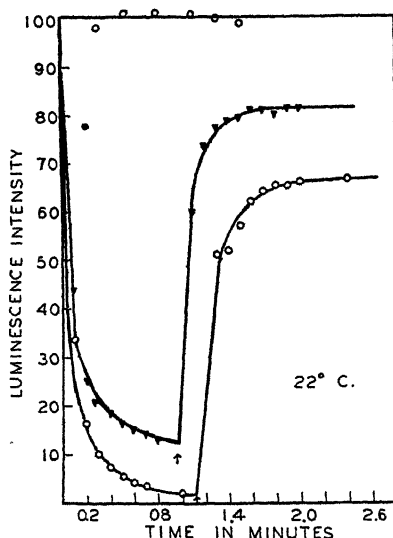


FIG. 9. The reversibility of the effect of strong alkali on the intensity of luminescence at 22° C. After introducing the alkali, at zero time, equivalent acid was added, at the time indicated by the arrows, to restore the original pH of approximately 7.

occurs, and the intensity at other respective pH as  $I_2$ . Using equation (9) or (10), we find that the  $\log \left( \frac{I_1}{I_2} - 1 \right)$  plotted against pH throughout the acid range between 3.6 and 6.4 results in a straight line of slope approximately 1 (Fig. 10). Assuming that the site of these effects of hydrogen ions is on the luciferase molecule, possibly on the luciferin as a prosthetic group, the slope of this line indicates that a ratio of one hydrogen ion to one luciferase molecule is involved in the equilibrium. Similar results are obtained in the corresponding analysis with respect to hydroxyl ions, throughout the alkaline range between pH 7 and pH 9, although a complication is encountered in the denaturation of the protein that occurs increasingly fast with rise in alkalinity, as well as temperature.

The relation between pH and luminescence intensity, throughout the range in which the enzyme does not undergo appreciable destruction, may be expressed by a single equation which assumes that one hydrogen or one hydroxyl ion per enzyme molecule is required to eliminate catalytic activity. Using  $K_5$  to designate the constant for the equilibrium between hydrogen ions and the enzyme, and  $K_6$  similarly for the hydroxyl ions, the values of these constants

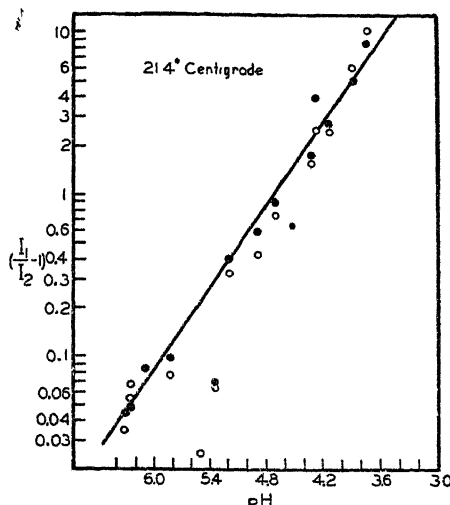


FIG. 10. An analysis of the data in Fig. 8, and similar experiments, as a Type I inhibition. The solid and open circles represent the intensity of luminescence 1 and 2 minutes, respectively, after adding the acid or alkali. The slope of the line is approximately one. Semilogarithmic scale.

may be estimated from the data available in Figs. 7 and 8. We thus arrive at the following equation:

$$\left(\frac{I_1}{I_2} - 1\right) = K_5 (\text{H}^+) + K_6 (\text{OH}^-) = 4.84 \times 10^4 (\text{H}^+) + 4.8 \times 10^6 (\text{OH}^-) \quad (11)$$

In Fig. 11 the theoretical curve has been plotted for this equation. The points are from the experiments represented in Figs. 7 and 8.  $K_5$  and  $K_6$ , of course, vary with the acid and base used. This indicates that the companion ions of ( $\text{H}^+$ ) and ( $\text{OH}^-$ ) combine with the enzyme.

At the extremes of pH shown in Fig. 11 there is evidence of inhibitory effects greater than would be expected from the simple equation (11), thus indicating that additional reactions, unfavorable to luminescence, occur. On the whole, however, the agreement between the experimental data and theory is satisfactory, showing that the possible complicating factors are not so prominently concerned as to obscure the primary mechanism. Equations (9) and (10)

provide a basis for the understanding of these effects. According to the smooth curve of Fig. 11, and the reactions (4) and (4') assumed, the  $pK$  of ALH occurs at pH 4.95, and the  $pK$  of LH at 9.45.

(c) *The pH-Temperature Relation of Luminescence in the Acid Range*

At acid pH values which cause moderate and reversible diminutions in luminescence at the optimum temperature, the effect of the given pH changes greatly with change in temperature. At low temperatures the addition of acid results in a much greater inhibition than for the same pH at the higher

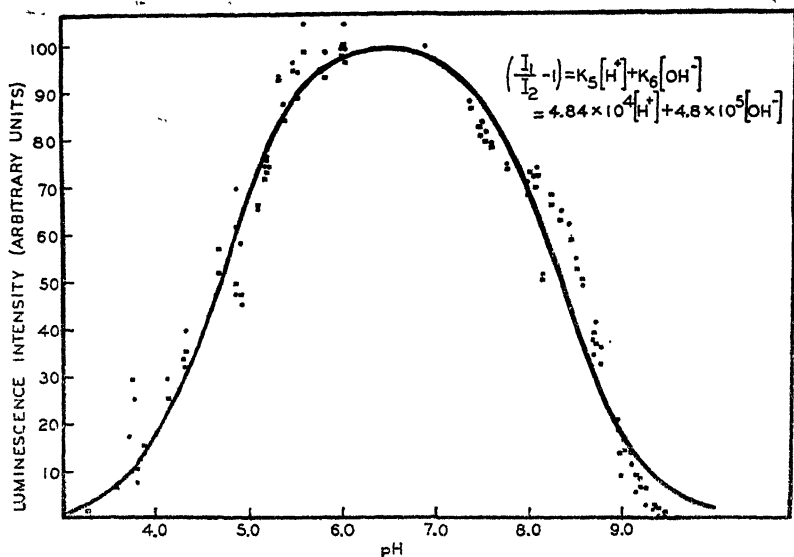


FIG. 11. Luminescence intensity *versus* pH, based on the intensity at 1 minute (squares) and 2 minutes (circles) after adding acid or alkali. The smooth curve has been drawn from the equation shown in the upper right hand margin of the figure.

temperatures (Fig. 12). Such a result is understandable in terms of the assumed reactions leading to luminescence. Thus, at low temperatures, relatively little of the ALH is dissociated (reaction 4), while at high temperatures the ALH may be assumed to be very largely dissociated. Thus, with hydrogen ions acting as the inhibitor, a rise in temperature decreases the inhibition by dissociating the inhibitory agent, just as in the case of sulfanilamide, which appears to act in very much the same manner. The action of sulfanilamide will be considered in more detail presently. The action of hydrogen ions may be considered either from the point of view of inhibitors in general, or from the point of view of the mechanisms controlling the activation energy for luminescence. The latter point of view is taken first, in the following paragraph.

The relation between the activation energy for luminescence and the pH of the medium<sup>6</sup> is evident in Fig. 12. At the pH of 5.05 the activation energy, calculated as a straight line relation over the range between 2° and 7° C., amounts to 40,900 calories, while at pH 6.92 it amounts to approximately half of this value, or 20,700, calculated in a similar manner. In both cases the heat of ionization constitutes a definite fraction of the over-all activation energy. At the optimum temperature and at neutrality, the ionization of ALH to  $AL^-$  must be nearly complete, as shown by the fact that  $K_i$  ( $H^+$ ), which measures the concentration of (ALH), is zero. At the optimum tempera-

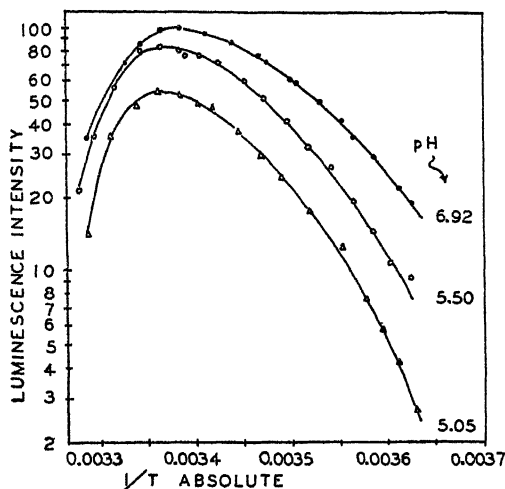


FIG. 12. The intensity-temperature relation of luminescence at acid pH values (*P. phosphoreum*). The activation energy for luminescence, indicated by the slopes of the lines at low temperatures, amounts to approximately 20,700 calories at pH 5.05 and 40,900 calories at pH 6.92. The difference of about 20,000 calories is attributed to the heat of ionization of the ALH in the process of activation. Semi-logarithmic scale.

ture and pH, however, the value of  $K_1$ , the equilibrium constant governing the reversible protein denaturation of the enzyme, is sufficiently large to cut down the apparent, or observed, rate of the luminescent reaction. Since the value of  $K_1$  under given conditions can be only approximately determined, it becomes difficult to estimate with accuracy just what fraction of the change in the apparent activation energy is to be attributed to the heat of ionization, and what fraction to the change in  $K_1$  with temperature. At low temperatures, the difference of about 20,000 calories between the activation energies at

<sup>6</sup> The pH indicated in connection with the data shown in Figs. 12 and 15 are the pH values as determined at room temperature with the Beckman glass electrode pH meter.



pH 5.05 and pH 6.92 is clearly to be attributed largely to the ionization process, and by this route we are able to arrive at an estimate for the heat of ionization which checks roughly with the 14,000 calories heat of dissociation of ALH, when the hydrogen ions are considered as an inhibitor and analyzed in the appropriate manner (Fig. 13).

In Fig. 12, the influence of dissociation on the activation energy for luminescence is evident in two distinct trends of the curves. In the first place, at low temperatures, the slope is steeper at an acid pH, which requires more energy for ionization. In the second place, with any pH, the slope decreases

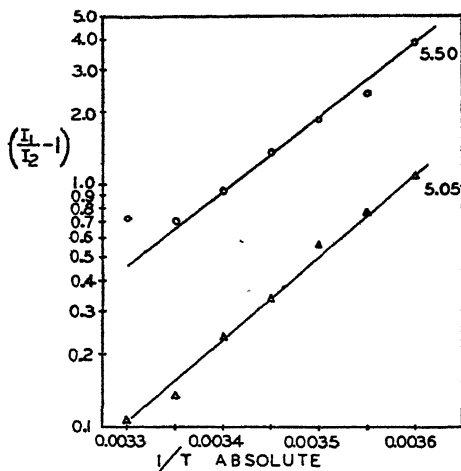


FIG. 13. Analysis of the data shown in Fig. 12, considering hydrogen ions as a Type I inhibitor. The slopes of these lines indicate a heat of reaction of about 14,000 calories for the equilibrium involving hydrogen ions. The 14,000 calories check roughly with the 20,000 calories indicated for the same process as judged by the slopes of the lines in Fig. 12. Semilogarithmic scale.

in going from the low temperatures toward the optimum. A part of this decrease in slope is brought about, according to our theory, by the increasing proportion of ionized over unionized ALH. In approaching the optimum, however, an additional factor becomes more and more significant in decreasing the slope of the curve; *viz.*, the increasing value of  $K_1$  with rise in temperature in the denaturation equilibrium. Qualitatively, the picture is easily understandable on the basis of what has already been set forth. Quantitatively, it would be difficult to distinguish with accuracy all of the number of factors or reactions which operate to influence the apparent activation energy and heats of denaturation equilibria. Results obtained in experiments with hydrostatic pressure, described below, are consistent with the foregoing partly qualitative interpretations.

*(d) The Relation of Luminescence to pH and Pressure at Constant Temperature*

Fig. 14 shows the relation between luminescence intensity, hydrostatic pressure, and pH at a single constant temperature. At this temperature, as previously shown (Brown, Johnson, and Marsland, 1942) pressure up to some 500 atmospheres or more has little effect on luminescence at pH 7.3, although at lower temperatures pressure reduces, and at high temperatures pressure increases the intensity at the same pH. As the reaction of the medium is made more acid a pressure effect becomes more noticeable, acting in the direction of retarding luminescence. In connection with Fig. 14 it should be borne

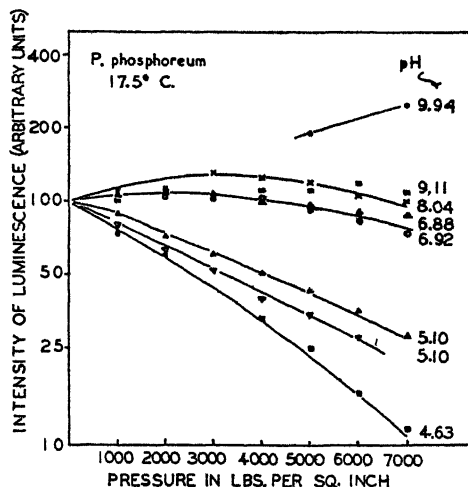


FIG. 14. The relation between the observed intensity of luminescence and hydrostatic pressure at various pH values at 17.5° C. At each pH, the intensity at normal pressure has been arbitrarily taken as equal to one hundred. The actual intensities at the different pH and at normal pressure, vary in the manner indicated by Fig. 11.

in mind that the actual intensity at different pH, particularly at the extremes, may be very different, although in this figure the intensity at normal pressure has arbitrarily been taken as 100 in each case, in order to show more clearly the effect of pressure alone. Thus, in a medium whose pH is 5.10, pressure readily reduces the intensity of luminescence, just as it does at a neutral pH at low temperatures. In both cases the ALH is relatively unionized, and when the ionization is increased by a rise in temperature, with the neutral solutions, or by a decrease in hydrogen ions at optimum temperature, the pressure effect largely disappears. It is evident, therefore, that the ionization is accompanied by a considerable volume change, amounting to about 71 cc. per gm. molecule as a mean value from the slopes of the duplicate determinations for pH 5.10, shown in Fig. 14. At low temperatures and neutral pH, the volume change of

activation, calculated earlier (Brown, Johnson, and Marsland, 1942) appeared to be 58 cc. at 0° C. The calculations of Eyring and Magee (1942) indicated 50 cc. at 0° C., and showed a temperature dependence for this  $\Delta V^\ddagger$ <sup>7</sup> which can now be accounted for in part by the temperature dependence of the degree of ionization of ALH; *i.e.*, a strong effect of pressure in reducing the intensity of luminescence will be noted wherever the conditions are unfavorable for ionization of the ALH. Factors which increase this ionization might be expected to counteract the pressure diminution of luminescence.

It will be recalled that pressure also counteracts the reversible denaturation of the protein, thereby increasing the intensity of luminescence whenever the value of  $K_1$  becomes significant. Thus, the disappearance of a pressure effect on luminescence in the neighborhood of the optimum temperature and in neutral solutions is due to two influences, *i.e.* the effects on  $K_1$  and  $k_2$ , and for reasons already indicated it would be difficult to state much better than has been done previously (Eyring and Magee, 1942) the quantitative rôle of each in the total effect observed. The data indicate that the volume change accompanying ionization is largely responsible for the effect of pressure in increasing the activation energy.

At increasingly acid pH, the pressure effect tended to become less and less readily reversible on release of pressure. At the quite acid pH of 4.63 the pressure diminution of luminescence became slightly greater with increase in pressure, and the effects were not appreciably reversible, on release of pressure, over short periods of observation. The irreversible, or only slowly reversible effects are possibly in the protein portion of the enzyme, and may be similar to the changes which take place when the viability of bacteria is reduced and proteins are permanently denatured by high pressures (Hite, Giddings, and Weakley, 1914; Larson, Hartwell, and Diehl, 1918; Giddings, Allard, and Hite, 1929; Bridgeman, 1931; Cattell, 1936; Matthews, Dow, and Anderson, 1940.

One further point calls for mention in connection with Fig. 14. It is apparent that although pressure causes no appreciable increase in brightness at this temperature and slightly alkaline reactions, pronounced increases may occur at the same temperature but in extremely alkaline media. At pH 9.94 the luminescence was fairly rapidly and irreversibly extinguished, but the application of pressure immediately increased the intensity at that moment. The irreversible destruction of luminescence by alkali will be discussed later. The point of present interest is that the increase in luminescence under the influence of pressure in quite alkaline solution appears to be the result of a pressure effect on the process of protein denaturation, rather than any effect on the

<sup>7</sup> In accordance with the notation usually employed in connection with the Theory of absolute reaction rates, the double dagger ( $\ddagger$ ) is used to indicate the  $\Delta F^\ddagger$ ,  $\Delta H^\ddagger$ ,  $\Delta E^\ddagger$ ,  $\Delta V^\ddagger$ , and  $\Delta S^\ddagger$ , respectively, in the equilibrium between the normal and activated states of reactants in a rate process, in distinction to the usual thermodynamic constants of equilibria between initial and final states.

dissociation of ALH. It would seem that, in general, the protein of the enzyme is stable in acid solution, but undergoes denaturation in alkaline solution. Some highly purified proteins have been found to share this characteristic (Northrop, 1939).

(e) *The pH-Temperature Relation of Luminescence in the Alkaline Range*

The intensity of luminescence in relation to temperature is shown in Fig. 15 for suspensions in NaCl solutions buffered by phosphate over the range

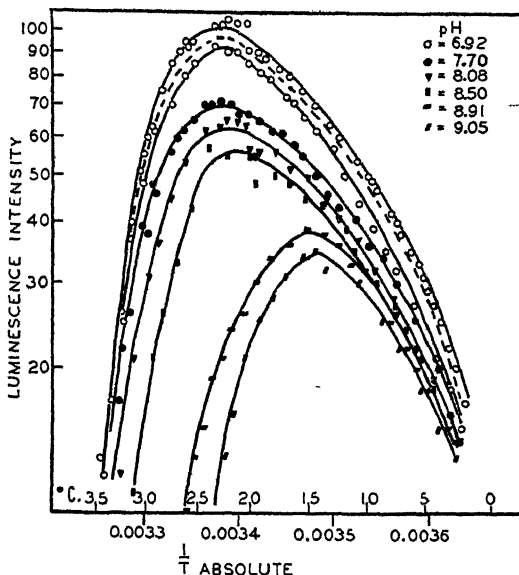


FIG. 15. The intensity-temperature relationship of luminescence at alkaline pH values. The duplicate curves drawn through the open circles were obtained with portions of the same suspension about  $1\frac{1}{2}$  hours apart. The dotted line was taken as the mean value in analyzing the data with respect to temperature (Fig. 16). Semi-logarithmic scale.

between pH 7 and 9. In general the inhibition is less at low temperatures. At reactions more alkaline than pH 8, there is a marked tendency for the maximum intensity of luminescence to occur at lower temperatures. From the relation of hydroxyl ions to the luminescent reaction as already discussed, it would follow that the initial effect of increasingly alkaline reactions, beyond a pH of 7.0, consists in an effect on the LH, slowing the light-emitting reaction. This effect itself will have a temperature coefficient, inasmuch as changes in temperature will influence the degree of ionization of the reactants taking part in luminescence.

In addition to the effect of the OH ions on the LH of reaction (5), the protein of the enzyme at optimum temperatures undergoes a reversible denaturation

at high alkalinities, as shown by the action of pressure. Thus, two distinct reactions are affected by increasing concentrations of hydroxyl ions, both in the direction of decreasing the intensity of luminescence. The first effect, on the ionization of essential reactants, takes place most prominently between pH 7.0 and pH 8.5. Beyond a pH of 8.5, the same reaction is still affected, but is not responsible for the major fraction of the further inhibition, which is now largely due to the denaturation of the protein. Accompanying the reversible phase of this denaturation is the irreversible denaturation. Thus, if we exclude the not altogether unlikely possibility that the protein of more than one enzyme involved in luminescence is also affected, there are at least three reactions which are influenced in the same direction of inhibiting luminescence by increasing the alkalinity beyond pH 7.0, namely, the ionization of LH, the reversible protein denaturation, and the irreversible protein denaturation. A possible source of error in the opposite direction conceivably takes place at the high temperatures. Bodine and Allen (1938) have shown that brief exposure to high temperatures, or to any of several protein denaturants, results in an activation of certain oxidative enzymes. Some indication that a similar effect might occur in the luminescent system was encountered in observations on the reversibility of the high-temperature inhibition of luminescence in alkaline solutions. For it was frequently found that when a suspension was heated briefly to a temperature well beyond the optimum, thereby diminishing the luminescence to around 10 per cent, it would then return, on cooling quickly, to an intensity somewhat higher than its previous maximum. The true significance of this increase is not fully clear on the basis of the data at hand, and it apparently does not constitute an important source of error. It is extremely unlikely that the reversibility of the temperature diminution of luminescence results from an almost perfect balance between an activation and an irreversible denaturation of the total number of enzyme molecules.

With the various sources of error and possible complications pointed out above, it is obvious that an analysis of the data in a family of curves, such as those given in Fig. 15, can only be approximate. The formulations that have been derived for relatively simple situations are useful, first of all, in showing whether or not the measured process is too complicated to analyze in such manner. If not, the formulations are useful in estimating the heats of reaction, etc., with an accuracy depending on both the precision of the data and the extent to which it is complicated by other reactions. Fig. 16 gives the temperature analysis for the curves in Fig. 15, and shows that the situation is indeed complicated by more than one reaction. The prominent up-turning of the curves in the high-temperature range, especially in the case of the high pH also, must represent multiple inhibitory reactions. At the lower temperatures, where the actual inhibition caused by a given  $\text{OH}^-$  concentration is much less, the lines become straight and parallel. There is a tendency, of course, for the

lines to become straight at low temperatures, where the values of  $1/K_1$  become extremely large in comparison with the other terms in the expression used for calculating the points on the ordinate. The slope of the lines, however, is determined both by the equilibrium constant  $K_1$ , and by the value of the expression  $\left(\frac{I_1}{I_2} - 1\right)$  at each temperature. The slopes of the straight line portions of the curves in Fig. 16 indicate a rather large heat of reaction, of about 60,000 calories. Most of this heat represents the  $\Delta H$  of the reversible denaturation of the protein, while a much smaller fraction is to be attributed to the heat of ionization in the luminescent reaction (5). The mechanism of denaturation by hydroxyl ions will be referred to again in connection with the action of urethane and alcohol.

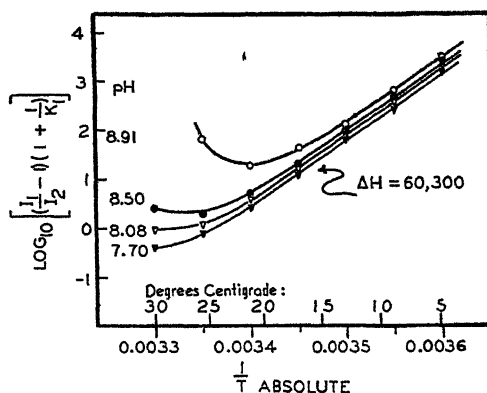


FIG. 16. Analysis of the data shown in Fig. 15

## V. THE ACTION OF SULFANILAMIDE

### (a) *Inhibition in Relation to Concentration and Temperature*

Previous studies have already indicated that the sulfanilamide inhibition of luminescence is of the kind we have termed "Type I." Equation (9) applies, and since the combination of enzyme and inhibitor evidently evolves heat, the inhibition will in general decrease with rise in temperature and concomitant dissociation of the enzyme-inhibitor complex. At temperatures beyond that of the normal optimum, where the reversible denaturation equilibrium constant  $K_1$  rapidly increases and brings about increasing diminutions in the over-all intensity of luminescence, a practically complete dissociation of moderate concentrations of the inhibitor may occur. The nature of these effects is such that a slight shift of the temperature of maximum luminescence takes place in the direction of the higher temperatures. A simple differential equation has been derived to estimate the amount of this shift (Johnson and Eyring

1943) but the calculations yield values which are considerably too high for the results obtained in experiments, *i.e.* the actual shift in temperature of the maximum is less than that which would be predicted, given the constants  $K_1$  and  $K_2$ , and the concentration of inhibitor added. A part of the error undoubtedly arises from the sensitivity of the equation to experimental error, and the difficulty of obtaining sufficiently precise values for the equilibrium constants  $K_1$  and  $K_2$  respectively. Probably the largest error, however, is due to over simplification of the equation, which does not take into account all the factors which may influence the observed result. For example, the changing tempera-

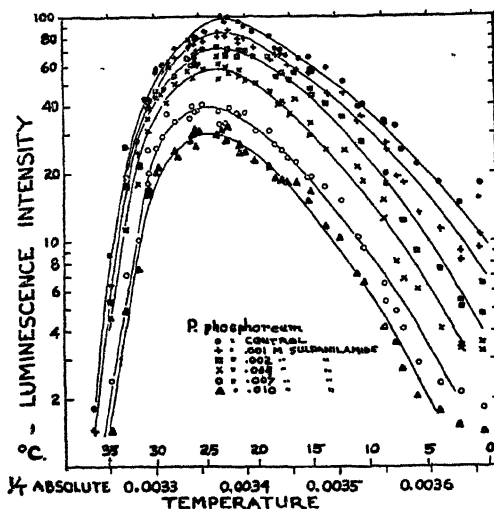


FIG. 17. The intensity-temperature relationship of luminescence in corresponding portions of the same suspension of *P. phosphoreum* containing different amounts of sulfanilamide as indicated. Note the tendency of maximum luminescence to occur at slightly higher temperatures with increasing concentrations of sulfanilamide. Semilogarithmic scale.

ture coefficient of activation energy, resulting from the varying degrees of ionization at different temperatures, was not taken into account. Thus, while the picture is again qualitatively clear with reasonable certainty, some of the quantitative predictions relating to the more detailed aspects are difficult to make with satisfactory accuracy.

Figs. 17 and 18 show the results of an experiment with portions of a single suspension of cells in which the effects of several concentrations of sulfanilamide were measured over a wide temperature range. The family of curves in these figures may be used as the basis for analyzing both the relation between concentration of sulfanilamide and inhibition of luminescence at constant temperature and inhibition at a constant concentration of sulfanilamide. The results

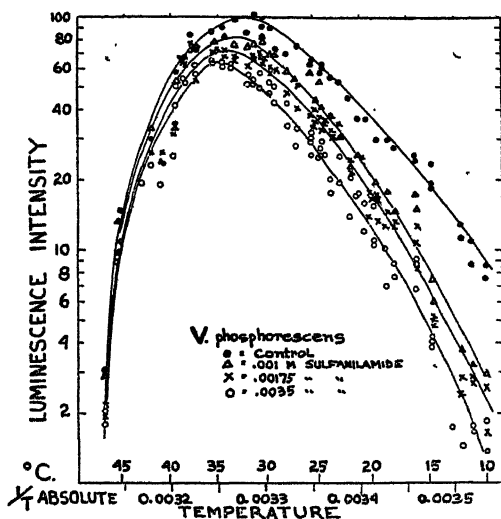


FIG. 18. The intensity-temperature relationship of luminescence in corresponding portions of the same suspension of *Vibrio phosphorescens* containing different amounts of sulfanilamide as indicated. Note the different position of the normal maximum intensity of luminescence in this species, as compared to that of *P. phosphoreum*, with respect to absolute temperature. A similar change in the temperature of maximum luminescence, however, is caused by the addition of sulfanilamide.

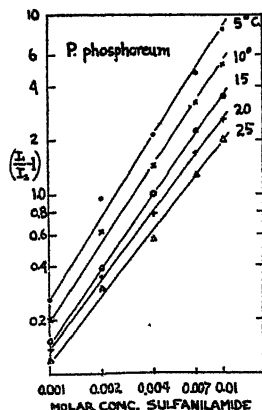


FIG. 19. Analysis of the data shown in Fig. 17 with respect to the concentration of sulfanilamide at different given temperatures. The slopes of the lines lie between 1 and 1.5. Log-log scale.

of these analyses for Fig. 17 are given in Figs. 19 and 20, respectively. The slopes of the lines in Fig. 19 vary only slightly with temperature and indicate that the ratio of sulfanilamide-enzyme molecules is somewhat greater than one.



In many experiments dealing with the effects of concentration alone, however, the slope was found to be very nearly equal to one. It would appear, therefore, that while complicating factors and experimental error give rise to some variation, the actual ratio may be considered to be one, just as in the case of hydrogen ions. Further resemblance between the effects of sulfanilamide and hydrogen ions is evident in the heats of reaction, calculated from the slopes of the curves in Fig. 20. These heats, which are strikingly similar throughout the range of sulfanilamide concentration of 0.001 to 0.01 molar, amount to

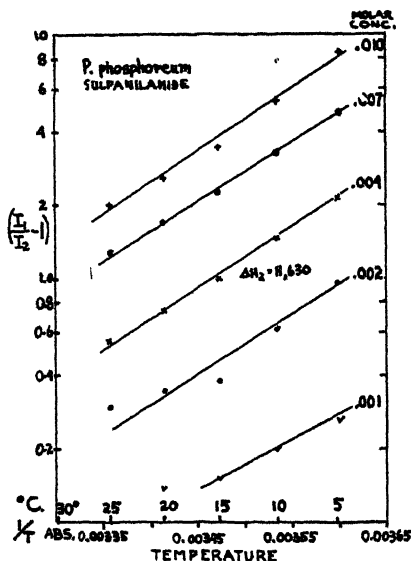
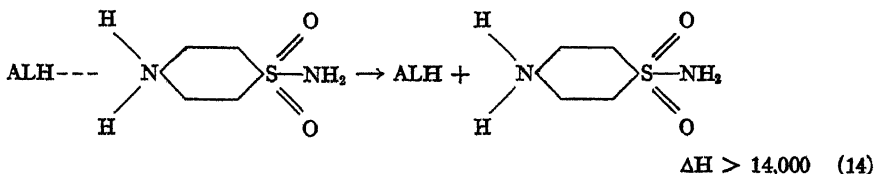
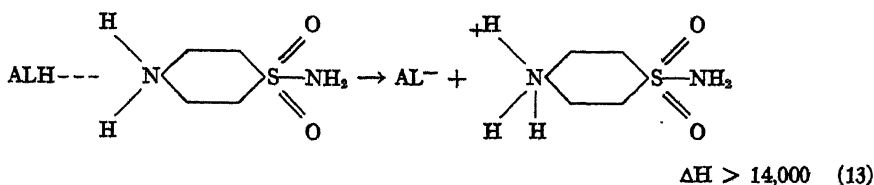
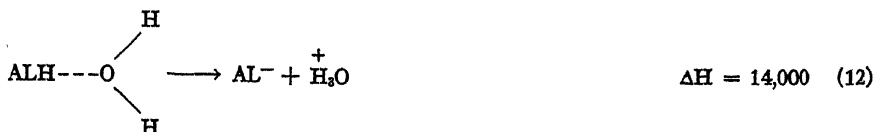


FIG. 20. Analysis of the data shown in Fig. 17 with respect to temperature for different given concentrations of sulfanilamide. The slopes of these lines indicate a heat of reaction of about 11,600 calories, as compared with 14,000 calories for hydrogen ions.

about 11,600 calories. This value is somewhat less than the average of 14,070 calories obtained by a similar analysis with hydrogen ions instead of sulfanilamide. Some previously reported estimates of the heat of reaction of the sulfanilamide equilibrium, based on the effects of one concentration of the drug at different temperatures in each of several species of luminous bacteria (Johnson, Eyring, and Williams, 1942), are higher than the present value of 14,070 calories for the hydrogen ion dissociation. Small differences in these heats of reaction are difficult to determine with certainty.

A reasonable explanation of the foregoing results is as follows: In the absence of sulfanilamide, reaction (4) yields the necessary  $AL^-$ , with an absorption of

14,000 calories. When sulfanilamide is added, it seeks out the ionizing hydrogen, and raises this ionization energy (*cf.* Equation 13) to a larger value, blocking ionization. This inhibits formation of  $AL^-$  and therefore light emission. Reactions (12) and (13) will presumably involve about the same volume change. The lack of a pressure effect on (14) shows this involves no volume change.



The effect of temperature in relation to either hydrogen ion formation (13) or to sulfanilamide dissociation (14) will be fundamentally the same, since in both cases the heat of reaction is largely that which is associated with the ionization.

#### (b) Relation to *p*-Aminobenzoic Acid and to Growth

Because of the very extensive study that has been made of *p*-aminobenzoic acid (PAB) in relation to the sulfanilamide inhibition of growth (Woods, 1940, and numerous subsequent papers) the action of this drug in luminescence assumes particular interest, especially if the proposed luminescence reaction scheme makes it possible to reach a somewhat clearer picture of the mechanism involved in the effects on growth.

With respect to growth, low concentrations of PAB and closely related derivatives are able to overcome relatively high concentrations of sulfanilamide, and this effect depends only on the ratio, not the absolute concentrations, of the two drugs. Moreover, the phenomenon is very general, occurring in all cases where sulfanilamide inhibits growth under otherwise favorable conditions for the organism concerned. The PAB antagonism of sulfanilamide is lessened, or eliminated, however, under certain conditions; *e.g.*, at temperatures

slightly above the optimum (Lee, Epstein, and Foley, 1943; Lee and Foley, 1943). It is evident that the two drugs under ordinary conditions, act "competitively" at the same site, a probability that is strengthened by steric considerations of the closely similar molecular configurations, and even more so because of their having amino groups of similar basicity. Thus, it is generally believed that PAB itself (or a closely related compound) is an essential substance in normal metabolism which is displaced by sulfanilamide, thereby leading to an inhibition in the rate of growth. On the grounds of this assumption, plus an experimentally demonstrated relation between the growth-inhibitory properties of various sulfonamide derivatives and the electro-negativity

TABLE II

*Luminescence Intensity, in Arbitrary Units, of P. phosphoreum and A. fischeri, Respectively, Suspended in Phosphate-Buffered NaCl Solution, pH 7.3, Containing Sulfanilamide Alone and in Addition to Various Concentrations of p-Aminobenzoic Acid (PAB). Room temperature, Approximately 20°C.*

Molar concentration of sulfanilamide	Controls: No PAB, no sulfanilamide (duplicates)		No PAB	0.001 molar PAB, times fraction indicated in addition to sulfanilamide										
				$\times 1$	$\frac{1}{2}$	$\frac{1}{3}$	$\frac{1}{4}$	$\frac{1}{5}$	$\frac{1}{6}$	$\frac{1}{7}$	$\frac{1}{8}$	$\frac{1}{9}$	$\frac{1}{10}$	
<i>A. fischeri</i>														
0.00015	22.7	20.	14.8	12.8	12.9	13.0	15.0	14.8	15.9	16.5	15.7	14.7	14.5	
0.00025	19.0	18.6	12.6	11.5	11.4	12.3	10.2	12.	10.4	11.6	12.	12.4	12.3	
0.00160	18.	18.2	4.9	4.4	4.3	4.1	4.8	4.4	4.3	4.4	4.5	4.1	4.3	
<i>P. phosphoreum</i>														
0.0025	21	19.2	11.5	10.7	13.5	9.5	11.7	12.5	10.8	10.4	11.6	11.7	10.5	
0.005	22	23.	8.6	8.4	7.8	7.3	7.4	8.7	9.1	8.1	8.3	8.6	8.6	
0.010	20.2	19.5	3.2	3.1	3.3	3.6	3.8	3.1	3.2	2.9	3.0	2.9	3.3	

of the sulfone group, Bell and Roblin (1942) have advanced a theory regarding the type of derivative which might be expected to inhibit growth in fundamentally the same manner as sulfanilamide.

In luminescence, as in growth, PAB and sulfanilamide both appear to act at the same site on the critical enzyme concerned but unlike the situation with respect to growth, PAB either has no effect on luminescence beyond the limits of experimental error, with or without sulfanilamide simultaneously present, or causes an inhibition (Table II; cf. also, Johnson and Chase, 1942; Johnson, Eyring, and Williams, 1942; Johnson, Eyring, and Kearns, 1943). The inhibitory concentration of PAB in luminescence is roughly 10 times the concentration of sulfanilamide needed to cause the same effect. Although, as numerous papers have shown, high concentrations of PAB will also inhibit growth, this

inhibition can hardly involve the same site through which PAB counteracts the sulfanilamide inhibition of growth. The PAB inhibition will be considered later.

In explanation of these phenomena, it appears that in luminescence, PAB and sulfanilamide go on the same position, and both block the enzyme reaction. In growth they also combine through their basic amino groups competitively (Bell and Roblin, 1942) and the sulfanilamide blocks growth while low concentrations of PAB permit growth (though not luminescence) even in the presence of sulfanilamide. In growth, we assume that each of these drugs combines through its amino group, with an ionizable hydrogen on the enzyme. This blocks ionization of the enzyme. However, PAB can overcome this effect by ionization of its carboxyl group, while sulfanilamide cannot. It therefore seems reasonable to look for the different effect of these drugs on growth in this circumstance. Bell and Roblin's theory deals with a complementary aspect of the problem; *i.e.*, the relative efficiency of drugs to form the addition compound between the basic amino group and the ionizable hydrogen and the enzyme. Their theory is not concerned with explaining why the PAB-enzyme compound permits growth. According to our view the ability of the drug-enzyme compound simply to ionize permits growth, whereas luminescence requires not only the ionization, but also the giving up of a particular electron which apparently neither the PAB-enzyme nor the sulfanilamide-enzyme complex can do.

Derivatives of PAB might be expected to exhibit varying degrees of either an antisulfanilamide action or a bacteriostatic effect of their own, according to their ability, first, to be adsorbed properly, and second, to prevent or to permit the functioning of the enzyme. This view is in accord with the data of Wyss *et al.* (1943).

From the foregoing considerations it seems evident that the sulfanilamide inhibitions of luminescence and growth do not take place on identical enzymes, although they may take place on very similar ones. Among a number of distantly as well as closely related species of luminous bacteria the relative sensitivity of the "growth system" and the "luminescence system" might be expected to vary considerably. Such a variation among different species does not necessarily result from a different equilibrium constant for the enzyme-sulfanilamide complex in growth, or in luminescence, respectively. There is evidence to show that the sulfanilamide-luciferase equilibrium is characterized by closely similar heats and entropies of reactions among several species of luminous bacteria (Johnson, Eyring, and Williams, 1942). The observed differences in sensitivity among different species—that is, the wide differences in per cent inhibition of luminescence by a given concentration of the drug acting on different species under the same conditions of temperature, pressure, pH, etc.—may be accounted for purely on the basis of the relation of the sul-

fanilamide equilibrium to the normal, specific temperature-velocity curve of the organisms concerned. Thus, the equilibrium between sulfanilamide and the growth enzyme among different species likewise may be identical, involving the same bonds, in all cases, while the specific susceptibility in any one case depends not only on the particular relation between temperature, etc., and rate of reaction of the growth enzyme in the organism concerned, but also the possible production of metabolites which, like *p*-aminobenzoic acid, may displace sulfanilamide and substitute for the normal group whose functional activity has been abolished.

The general effect of sulfanilamide on the growth of luminous bacteria, together with observations on the luminescence of the cultures that develop

TABLE III

*Growth (G) and Luminescence (L) of Broth Cultures of Different Species of Luminous Bacteria in Relation to Sulfanilamide Concentration*

Molar concentration of sulfanilamide	<i>P. phosphoreum</i> *		<i>P. sepiac</i>		<i>P. splendendum</i>		<i>A. fischeri</i>		<i>A. harveyi</i>		<i>P. pierantoni</i>		<i>V. albensis</i>		<i>V. phosphorescens</i>	
	G	L	G	L	G	L	G	L	G	L	G	L	G	L	G	L
0.04	—	—	+	—	+	—	+	—	+	—	—	—	—	—	—	—
0.02	+	—	++	—	++	—	++	—	++	—	—	—	—	—	—	—
0.01	+	+	++	—	++	—	++	—	++	—	—	—	—	—	—	—
0.005	+	++++	++	+	++	—	++++	—	++++	—	—	—	—	—	—	—
0.0025	++	++++	++++	++	++	—	++++	—	++++	—	+	—	—	—	—	—
0.00125	++	++++	++++	++++	++++	+	++++	—	++++	—	+	—	++	+	+	—
0.00063	++	++++	++++	++++	++++	+	++++	—	++++	—	++	+	++	++	++	++++
0.00032	++	++++	++++	++++	++++	++++	++++	+	++++	+	++++	+	++++	++++	++	++++
0.00016	++	++	++++	++++	++++	++++	++++	++	++++	++	++++	++	++++	++++	++++	++++
0.00008	++	+	++++	++++	++++	++++	++++	++	++++	++	++++	++	++++	++++	++++	++++
0.0	++	+	++++	++++	++++	++++	++++	++	++++	++	++++	++	++++	++++	++++	++++

\* Incubated 53 hours at 15° C. All other species incubated 28 to 30 hours at 25° C.

in various concentrations of sulfanilamide, is summarized in Table III for all the well authenticated species available in this investigation.<sup>8,9</sup> It will be noted that in some cases abundant growth took place over a wide range of concentration of sulfanilamide which prevented luminescence. In other cases, the appearance of visible growth was accompanied by visible luminescence.

<sup>8</sup> These studies were aided by a grant from the Penrose Fund of the American Philosophical Society.

<sup>9</sup> Cultures of the species listed under the genus *Photobacterium* were obtained in 1939 from the Delft Collection, through the kindness of Professor A. J. Klyuyver. The culture of *Vibrio albensis* was obtained from the same source. This culture was almost indistinguishable from that of *V. phosphorescens*, kindly supplied from the collection of Professor Malcolm Soule. The species given under the genus *Achromobacter* are identical with the ones studied bacteriologically by Johnson and Shunk (1936).

Thus, among different species the systems limiting growth and luminescence respectively evidently have different thresholds of susceptibility to sulfanilamide. The susceptibility, of course, may rest upon complex factors, as indicated in the previous paragraph.

The relation of PAB to the sulfanilamide inhibition of growth of luminous bacteria is illustrated for *Vibrio phosphorescens* in Table IV. The effects appear to be fundamentally the same as those generally encountered among non-luminous bacteria; i.e., relatively small amounts of PAB will overcome the growth inhibition resulting from much larger concentrations of sulfanilamide. Furthermore, once growth took place, the cells

TABLE IV

*Relation between p-Aminobenzoic Acid and Sulfanilamide in the Inhibition of Growth (G) and Luminescence (L) of Broth Cultures of Vibrio phosphorescens at 25° C.*

Concentration of PAB molar	0.01		0.005		0.0025		0.00125		0.000625		0.00031		0.00015		0	
	G	L	G	L	G	L	G	L	G	L	G	L	G	L	G	L
0.04	-	-	-	-	-	-	-	-	±	-	-	-	-	-	-	-
0.02	-	-	++	-	++	-	++	-	++	-	+++	-	+++	-	-	-
0.01	++	-	++	-	++	-	++	-	++++	+	++++	+++	++++	++	-	-
0.005	++	-	++	-	++	-	++	-	++++	++	++++	++++	++++	++++	-	-
0.0025	±	-	-	-	++	-	++	-	++++	+++	++++	++++	++++	++++	-	-
0.00125	+	-	++	-	++	-	++	-	++++	++++	++++	++++	++++	++++	+	+
0.00062	+	-	+++	-	++	-	++	-	++++	++++	++++	++++	++++	++++	++	++
0.00031	+	-	++	-	++	-	++	-	++	+	++++	++++	++++	++++	++	++++
0.00015	±	-	++	-	++	-	++	-	++++	++++	++++	++++	++++	++++	++	++++
0.0	+	-	++	-	++	-	++	-	++++	++++	++++	++++	++++	++++	++	++++

were either luminous or non-luminous, in the different concentrations of sulfanilamide, in much the manner that would be expected from Table III. These results again point to the conclusion that the enzyme systems limiting the two processes, growth and luminescence, are not identical.

The inhibitory effect of PAB on growth is also apparent in Table IV. In fact, in high concentrations, PAB may not only inhibit growth by itself, but may show an additive effect with sulfanilamide. These inhibitory effects of PAB on growth must occur in some manner quite different from that by which this drug antagonizes the effects of sulfanilamide. In its inhibitory action PAB may combine either with a different enzyme essential to growth, and with relatively slight affinity for the drug, or with the same enzyme originally involved, but at a different site. Although no studies have yet been published that give sufficiently accurate and extensive data to enable an analysis of growth

in relation to temperature and concentration of the drug, on a basis similar to the one used in the present study with luminescence, there are experiments showing that, under given conditions, at temperatures above the normal "optimum," PAB tends to lose its capacity to antagonize sulfanilamide, and at high temperatures becomes increasingly inhibitory of itself (Lee, Epstein, and Foley, 1943). At a temperature above normal optimum, the growth-inhibiting potency of sulfanilamide, likewise, appears to increase greatly (White and Parker, 1938). At lower temperatures, there is a range in some cases through which the retardation of growth rates by sulfanilamide becomes less as the temperature is raised towards the optimum. Thus there is good reason to believe that both sulfanilamide and PAB act in a different manner at temperatures below and above the normal optimum. Moreover, the fact that the action of sulfanilamide at temperatures above the optimum evidently has a high temperature coefficient suggests that in this case the mechanism is similar to that of numerous protein-denaturing agents such as urethane, alcohol, and others, which will be discussed in detail in the next sections. Their inhibitions on a given system occur with a high temperature coefficient, and may be scarcely detectable, except in relatively high concentrations of the drug, at low temperatures.

Other significant aspects of the sulfanilamide inhibition of growth, and of its antagonism by PAB and other agents which act in a similar or different manner, can only be referred to briefly in this paper. The growth-stimulating effects of low concentrations of sulfanilamide, for example (Finklestone-Sayliss, Pain, and Patrick, 1937; Johnson, 1942; Lamanna, 1942; Green and Bielschowsky, 1942; Lamanna and Shapiro, 1943) take place by a mechanism that is not clear, but which reasonably involves the same type of phenomenon as the non-specific antagonism of sulfanilamide by urethane. The antagonism by urethane of the sulfonamide inhibition of luminescence has been considered quantitatively at some length (Johnson, Eyring, and Kearns, 1943). Qualitatively the same antagonism at appropriate temperatures and concentrations, has been demonstrated with respect to the growth of a number of common bacterial species in unpublished work in this laboratory.<sup>10</sup> These results suggest that a similar mechanism is involved in each case, namely, that of complex formation between urethane and sulfanilamide, thereby reducing the effective physiological activity of each when the two are simultaneously present.

Another important aspect, that of "drug-fastness," must involve still other mechanisms, which may or may not be associated with an increased production by the organisms of PAB-like metabolites (Landy, Larkum, Oswald, and Streightoff, 1943). With luminous bacteria, serial transfer of broth cultures in media containing increasing concentrations of sulfanilamide, resulted in an

<sup>10</sup> A number of experiments in this connection have been carried out in this laboratory by Mr. Perry G. M. Austin, Jr.; *cf.* also, Weinstein and McDonald, 1945.

increased resistance to sulfanilamide not only of growth, but also of luminescence, as shown by the data in Table V.

TABLE V

*Adaptation of Luminous Bacteria (A. fischeri) to Growth and Luminescence in Sulfanilamide-Containing Media*

Serial transfers at frequent intervals were made for several weeks in media in which sulfanilamide was gradually increased. Cross-inoculations were then made in all media. Growth and luminescence recorded after 1 day at 25°C. Data represent average of two duplicate tubes in each case.

Highest concentration of sulfanilamide in serial transfer	Growth (mm. galvanometer deflection in photocell densimeter)				Luminescence (arbitrary units)			
	Molar concentration of sulfanilamide in medium				Molar concentration of sulfanilamide in medium			
	0.0	0.003	0.005	0.01	0.0	0.003	0.005	0.01
0.0	25	12	8	11	5.5	—	—	—
0.003	21	12	9	10	1.9	<1.0	—	—
0.005	20	18	14	9	8.2	3.3	1.0	—
0.010	27	27	36	11	8.9	6.4	3.2	<1.0

## VI. TEMPERATURE DENATURATION OF THE ENZYME SYSTEM

### (a) *Relation between the Reversible and the Irreversible Denaturation*

By "denaturation" we mean the loss of one or more of the most characteristic properties of a given protein, usually occurring with an excessively high entropy and heat of reaction or activation energy, typical of this class of complex organic molecules (Eyring and Stearn, 1939). The actual changes that take place in the molecule in going from the "native" to the "denatured" state may be many and varied, and under different conditions the same molecular structure no doubt undergoes denaturation in somewhat different manners (Schmidt, 1944; Neurath, 1944). In certain cases the denaturation, e.g. as judged by the loss of catalytic activity, is quantitatively reversible in highly purified preparations of crystalline proteins (Anson and Mirsky, 1934). The stability of the denatured form which is in equilibrium with the native form varies with the conditions of pH, solvent, etc.

In the present instance, the decreasing intensity of luminescence above the optimum temperature takes place with the high entropy and heat of reaction characteristic of a protein denaturation, and has therefore been interpreted as such. The pressure effects indicate that it is accompanied by a large molecular volume increase. It is quantitatively reversible, when, under optimal conditions, the temperature is raised momentarily sufficiently high that the intensity of luminescence falls to as little as 1 or 2 per cent of its former maxi-



lum. At these higher temperatures, however, permanent destruction also rapidly occurs, with a high temperature coefficient. Thus, there is both a reversible and an irreversible phase to the denaturation process in bacterial luminescence. The purpose of the following experiments has been to clarify and elucidate somewhat the interrelation of these phases under both "normal" conditions and in the presence of certain inhibitors.

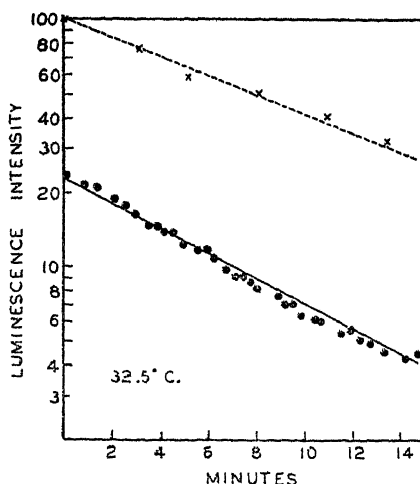


FIG. 21. The destruction of the luminescent system of *P. phosphoreum* at 32.5° C. roughly 10° above the normal optimum temperature for luminescence. The intensity at 22.5° C. and zero time has arbitrarily been taken as equal to one hundred. The crosses, through which the dotted line has been drawn, represent the intensity to which the luminescence returned when cooled from 32.5° C. to 22.5° C. at the time indicated. The experiment was carried out with eight to ten tubes, containing corresponding portions of the suspension, maintained at 32.5° C. Successive tubes were removed and cooled to obtain the points on the upper line. Note that the slopes of the two lines are almost parallel, indicating that most of the logarithmic decrease of luminescence with time at this temperature is not reversible with cooling. Semi-logarithmic scale.

Under the usual experimental conditions, bacterial luminescence remains constant for some time at the normal optimum temperature. At higher temperatures it undergoes a more or less rapid, logarithmic decrease with time. This decrease, that takes place with time at the higher temperatures, is almost entirely irreversible, as shown by Fig. 21. When portions of a suspension maintained at a constant temperature well above the optimum are cooled back to the optimum the extent of reversibility decreases almost in proportion with the time decrease. The lines are not precisely parallel in Fig. 21, for the slope of the upper line is somewhat less than that of the lower, as if the

amount of permanent destruction were somewhat less than the decrease with time would indicate. This recalls the possibility that some of the enzyme molecules, or their precursors, might be activated by above optimum temperatures, by  $\text{OH}^-$  ions, etc., which were mentioned in (IV,  $\epsilon$ ). The lines are so nearly parallel, however, that the slope of lower line may be considered a fair index of the rate of irreversible denaturation. The error in such an assumption would amount to no more than the difference in the slopes, which is evidently only slight. At different constant temperatures, the slopes of the lines thus provide a basis for estimating the activation energy for the

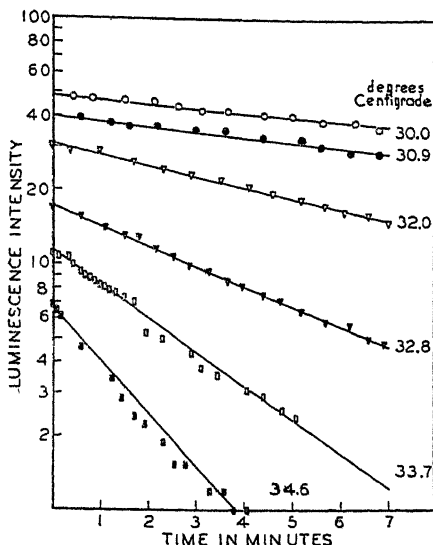


FIG. 22. The rate of destruction of the luminescent system of *P. phosphoreum* at temperatures above the normal optimum for luminescence. Semilogarithmic scale.

irreversible denaturation. Fig. 22 gives an example of the change in slopes with change in temperature. These slopes have been calculated in reciprocal seconds, and the logarithm of the resulting velocity constant has been plotted against the reciprocal of the absolute temperature in Fig. 23. The latter figure shows the results of three different experiments under similar conditions, carried out several months apart.

With regard to the significance of the data in Fig. 23, it will be noted that, in the first place, the points of the separate experiments lie fairly well on a straight line in each case, indicating that a first order reaction is taking place. Furthermore, these lines are all practically parallel, though there appears to be a slight difference in the intercept on the different occasions. The line in Fig. 23 was drawn by inspection, as representing the best average value

of the available data. The slope of this line would indicate that the irreversible denaturation has an activation energy of about 90,000 calories. Obviously, the activation energy could represent a reaction through which either the native or reversibly denatured enzyme, or both forms simultaneously, undergo destruction. Equation (15) gives the light intensity under these conditions. The notation is as follows:  $(A_0)$  = total luciferase;  $(A_{0i})$  = total initial luciferase when time ( $i$ ) equals zero;  $K_1$  = equilibrium constant between native and reversibly denatured luciferase;  $k_n$  = rate constant for the destruc-

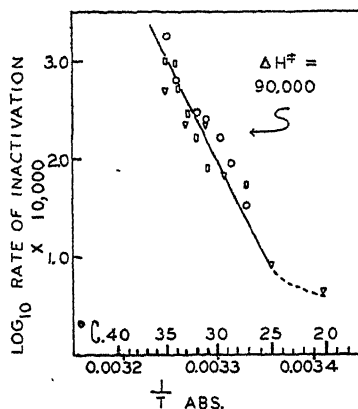


FIG. 23. The rate of destruction of the luminescent system, calculated in reciprocal seconds, from the slopes of the lines in Fig. 22 and similar experiments, as a function of temperature. The triangles, circles, and rectangles represent data from repeated experiments carried out several months apart. The point at 20° C., which falls too high, probably represents destruction through different reactions from those at the higher temperatures.

tion of native luciferase;  $k_d$  = rate constant for the destruction of the reversibly denatured luciferase; and the other symbols have their usual meaning.

$$I = \frac{bk(\text{LH}_2)(A_0)}{1 + K_1} = \frac{bk(\text{LH}_2)(A_{0i})}{1 + K_1} e^{-\left(\frac{k_n + k_d K_1}{1 + K_1}\right)t} \quad (15)$$

It is apparent from this equation that the slopes of the lines in Fig. 22 give the values for  $\left(\frac{k_n + k_d K_1}{1 + K_1}\right)$  at the respective temperatures. In Fig. 23, the logarithm of  $\left(\frac{k_n + k_d K_1}{1 + K_1}\right)$  is plotted against  $1/T$ . This is evidently a straight line relation over a range of almost 1000 in reaction rate constants. A straight line can be obtained over a wide range only if  $k_n = k_d$ . This, however, would mean that the reversible and the irreversible denaturation involve breaking different bonds. The point at low temperature in Fig. 23 connected

by a dotted line to the others involves complications due to the excessively long periods required to measure it. Since both denaturations involve extensive breaking of similar bonds it is to be expected that temperature, pressure, and reagents which promote one will promote the other, except for this divergence at below-optimum temperatures, referred to again in a later paragraph.

(b) *Relation to pH*

At optimum temperatures, or somewhat below, and with the experimental conditions ordinarily employed, the luminescent enzyme system appears to be stable throughout the acid range down to about pH 4.0. The pressure

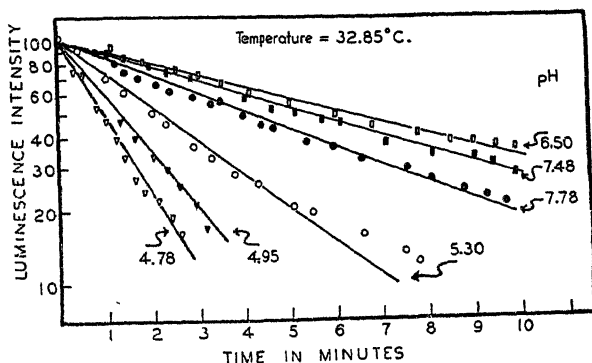


FIG. 24. The destruction of the luminescent system at 32.8°C. at different pH values. Semilogarithmic scale.

effect, referred to earlier, seems to indicate that the protein is stable in the alkaline range also up to about pH 9.0 at these temperatures. At temperatures above the optimum, however, the rate of the logarithmic decrease in luminescence with time varies markedly with pH, as shown in Fig. 24. When the velocity is calculated in reciprocal seconds from the slopes of these lines, and plotted as the logarithm against pH, the curve shown in Fig. 25 results. From this figure it is evident that there is a pH which is most favorable for the stability of the system, and this pH practically coincides with the pH of maximum luminescence at optimum temperature. It quite probably represents the isoelectric point of the protein. The data at hand, however, are not sufficient for a detailed interpretation of the denaturation process in relation to PH. The reaction is undoubtedly one which is subject to a complex interplay of various factors which require extensive investigation before a fully clear picture may be drawn. At present, it seems justified to conclude that the slope of the line at low pH values, in Fig. 25, provides an index, though somewhat rough, to the number of hydrogen ions concerned

in the denaturation at this temperature. A tangent to the curve in this region has a slope of 3.5, which may be considered as a first approximation of the number of hydrogen ions per protein molecule undergoing denaturation.

At low temperatures the luminescence system tends to be destroyed by reaction (5 $\gamma$ ), which gradually decomposes the luciferin, rather than the protein-enzyme. This type of slow destruction is very likely related to the phenomena studied at length in an earlier investigation (Johnson, 1939) and a different reaction from that involving the protein itself. According to this view, in Fig. 23, the point which, at lowest temperatures, falls too high for the straight line at higher temperatures, possibly represents such a destruction of luciferin.

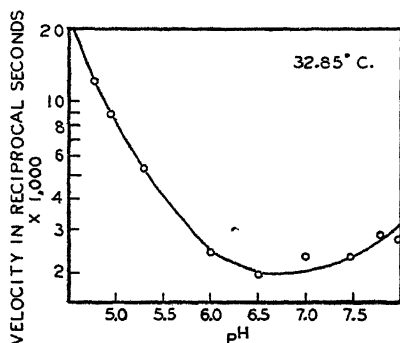


FIG. 25. The velocity of destruction at 32.8° C. as a function of pH, computed from the slopes of the lines in Fig. 24. Semilogarithmic scale.

### (c) Pressure versus Rate of Denaturation

The influence of hydrostatic pressure on the reversible denaturation has already been considered at some length in the previous publications, and has been amplified, with reference to the importance of hydrogen ion concentration, in the present work. At a favorable pH, pressures up to between 500 and 600 atmospheres appear to cause little destruction, since the effects are readily reversible. At the same pH, but at temperatures causing a progressive destruction of luminescence, the application of pressure will retard this irreversible denaturation. A volume increase of activation,  $\Delta V^\ddagger$ , therefore, must occur in this reaction. Data from experiments are shown in Figs. 26 and 27.

In Fig. 26 the decrease in luminescence with time at 34° C. and a pH of 7.3 is shown for different pressures. The slopes of these lines have been taken as a measure of the denaturation reaction rate, and the logarithm of the resultant velocity constants has been plotted against pressure in Fig. 27. Data from similar experiments at different constant temperatures are also included

in Fig. 27. It will be recalled that under the conditions of these experiments, luminescence first undergoes a considerable decrease in intensity through the reversible effects of above-optimum temperatures; *i.e.*, through influencing the value of the equilibrium constant,  $K_1$ . A further reduction then occurs with time, more or less rapidly according to both temperature and pressure, an increase in the former accelerating, and in the latter retarding this reduction, as shown in the figures.

Experimental difficulties, *e.g.* that of measuring by visual photometry a dim luminescence constantly decreasing in brightness, have made it impossi-

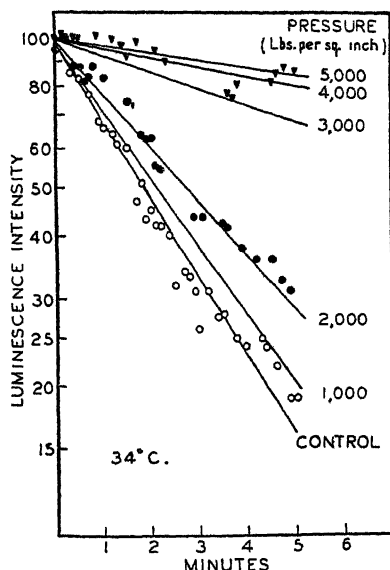


FIG. 26. The relation of the rate of destruction of the luminescent system to hydrostatic pressure. Semilogarithmic scale.

ble to obtain fully as accurate data with regard to the irreversible as with the reversible pressure-temperature effects. Thus the points appear somewhat scattered, but two relations appear evident in Fig. 27: (1) for pressures up to about 3000 pounds per square inch the lines are approximately parallel, and (2) at the higher pressures, particularly at higher temperatures also, there is a tendency for pressure to have less and less influence in slowing the net rate of the reaction.

In interpreting these relations, it seems clear in regard to (1) that the volume increase of activation is approximately the same over the range of temperature studied, covering a several-fold difference in reaction velocities. Taking the slope of the line for 32° C. as a measure of  $\Delta V^\ddagger$ , the data in Fig.

27 indicate that the volume increase amounts to about 71 cc. per gm. molecule in going from the normal to the activated state. This value is almost the same as that of 64.6 cc. at 35° C., calculated by Eyring and Magee (1942) for the volume increase in going from the initial to the final state in the reversible denaturation equilibrium.

As for the second of the above relations, the changing slope at higher pressures indicates among other things that the assumed picture is oversimplified, and that several reactions may be affected. In fact, with pressures higher than those shown in Fig. 27, it might be anticipated that a destructive action of pressure itself would become increasingly apparent, with the consequence that the over-all destructive process, as measured by luminescence alone,

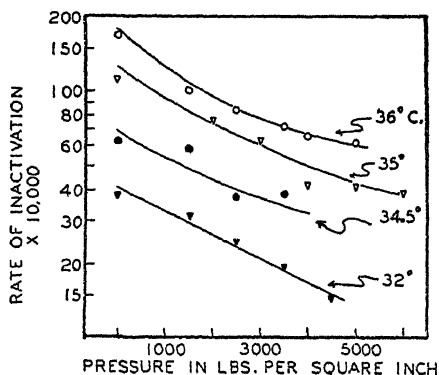


FIG. 27. The relation between the rate of destruction and hydrostatic pressure at various temperatures. The points in this figure were calculated from the slopes of the lines in experiments similar to Fig. 26. Semilogarithmic scale.

would become accelerated. In this event, of course, the curves would go through a minimum, of which there is already some evidence. Further studies with higher pressures would be necessary to resolve this question. The decrease in slope with increase in pressure is encountered again, and discussed, in connection with experiments concerning the alcohol inhibition of luminescence. An additional factor which should be taken into account for a more precise interpretation and calculation of the present results is the changing amount of  $A_n$  under the different conditions of temperature and pressure. For, while the slopes referred to above serve as a first approximation, the calculation of  $\Delta V^\ddagger$  should take into account  $k_n$  for different values of  $p$  and  $T$ . The data are sufficient to indicate clearly, however, that there is quite an appreciable volume change of activation in the irreversible denaturation reaction, and that this volume change is of the same order as that involved ordinarily in the reversible denaturation equilibrium.

## VII. THE ACTION OF URETHANE

(a) *The Time Course of Inhibitions by Type I and Type II Agents*

The straightness of the line in Fig. 23 leads to the conclusion, as noted earlier, that the reversible denaturation is independent of the irreversible one, although similar factors promote each. Thus the velocity,  $v$ , of denaturation can be written as  $v = k_n (A_0)$  where  $A_0$  is the sum of native and reversibly denatured luciferase concentration but not irreversibly denatured. The rate

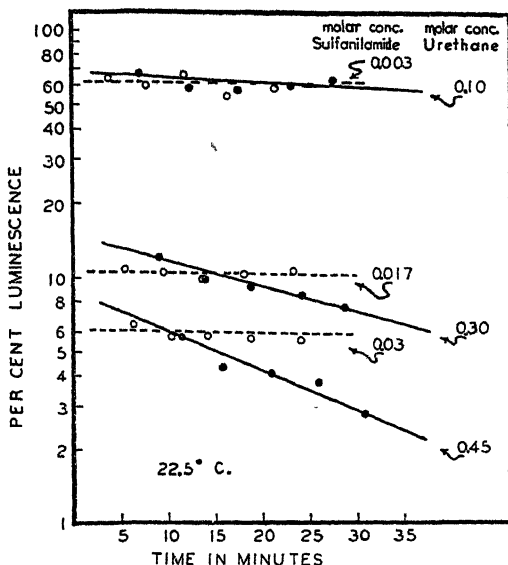


FIG. 28. Time course of inhibition by sulfanilamide and urethane respectively, in concentrations that cause initially similar diminutions in luminescence intensity. Semilogarithmic scale.

constant  $k_n$  will depend on temperature, pressure, and narcotics in much the same way as the equilibrium constant for reversible denaturation. This is because preliminary to the actual destruction of primary bonds there will be the same sort of opening of secondary bonds as occurs in reversible denaturation. Since Type I inhibitors, according to our view, combine with no bonds involved in reversible denaturation it is to be expected that they will not effect  $k_n$  of the irreversible denaturation. On the other hand Type II inhibitors, such as urethane in luminescence, break secondary bonds and so will increase  $k_n$ . Fig. 28 illustrates this effectiveness of urethane in speeding decomposition, and the absence of a similar effect of sulfanilamide. The results of the experiment in Fig. 28, were in fact anticipated. Although such a difference in the time course of inhibition by various



drugs acting on a given process has been frequently observed, the mechanism involved has, by and large, remained obscure (Quastel, 1943).

The slope of the line in Fig. 29 indicates that an average of one and a half more molecules of urethane are combined with the activated molecule undergoing irreversible denaturation than with the normal molecule.

With urethane and inhibitors of similar type a complication is likely to appear in the analysis of the relation between concentration and effect, as

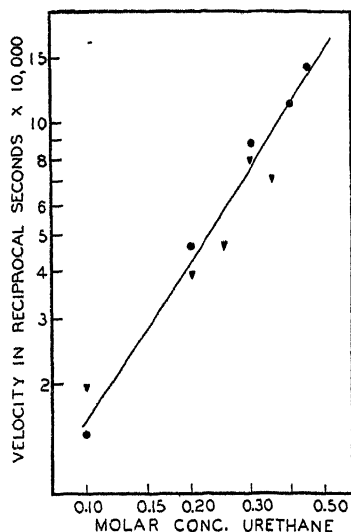


FIG. 29. Analysis of the data from two experiments similar to that shown in Fig. 28. The triangles and circles indicate repeated experiments carried out several months apart. The slope of this line is approximately 1.5. According to equation (10), this would indicate a ratio of about 1.5 between urethane and enzyme molecules in the destruction reaction concerned. Log-log scale.

well as temperature and effect, due to the superposition of an irreversible on a reversible denaturation. To some extent the irreversible reaction may be kept of minor importance by making quick observations, and an effort to this end has been made in carrying out the experiments described in the following discussion.

#### (b) Relation of the Inhibition to Temperature and Urethane Concentration

Figs. 30 and 31 give the relation between luminescence intensity and temperature, for two different species, *Vibrio phosphorescens* and *Photobacterium phosphoreum*, respectively, with various concentrations of urethane. The former of these species has a less brilliant luminescence, difficult to measure accurately. The points in Fig. 30 are, therefore, unusually scattered. The

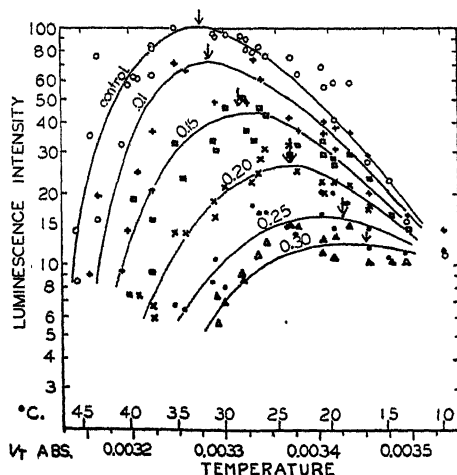


FIG. 30. The intensity-temperature relationship of luminescence in corresponding portions of a suspension of *V. phosphorescens* containing different concentrations of urethane. The relatively dim luminescence of this species is responsible for the unusually scattered points, determined by visual photometry. The smooth curves were drawn by inspection. The arrows indicate the calculated position of the optimum temperatures, according to the estimated equilibrium constants. Semi-logarithmic scale.

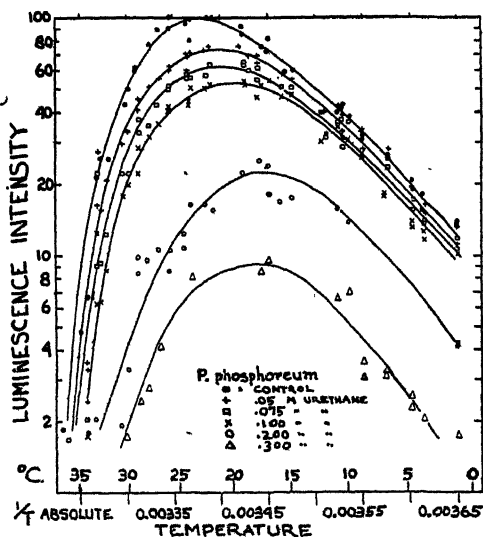


FIG. 31. The intensity-temperature relationship of luminescence in corresponding portions of a suspension of *P. phosphoreum* containing different concentrations of urethane. Semilogarithmic scale.

smooth curves have been drawn by inspection, and the analysis has been based on these lines.

At any given temperature, if the control without added urethane is taken  $I_1$ , and the urethane-containing suspension as  $I_2$ , then a plot of the logarithm of  $\left(\frac{I_1}{I_2} - 1\right)$  against the logarithm of molar concentration for different drug concentrations, gives a fairly straight line, as in the case of sulfanilamide, but with considerably steeper slope. Fig. 32 shows such a plot for several different temperatures, and it will be noted that the slopes increase with rise in

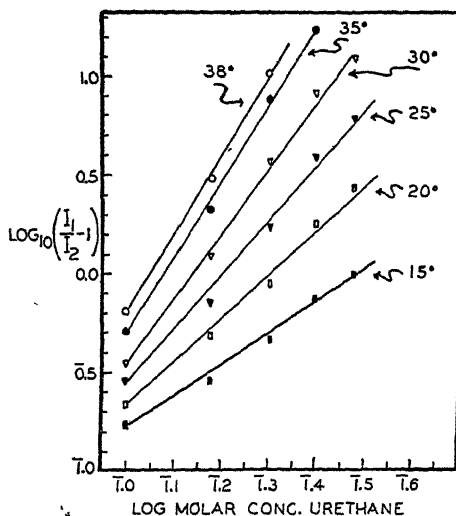


FIG. 32. Analysis of the relation between concentration and inhibition, at different temperatures, from the data given in Fig. 30. The significance of the increasing slopes of the lines with increasing temperature is discussed in the text.

temperature. Although the full explanation for the change in slope with rise in temperature cannot be given on the basis of the data available at present, certain points are worthy of brief discussion.

In the first place, the fact that the slopes of the lines in Fig. 32, as well as in numerous similar analyses, are not in all cases represented by whole numbers indicates the possibility that more than one type of combination takes place between the urethane and the protein. The observed slope would thus represent the average of two or more ratios of urethane-enzyme molecules, in the respective equilibria. Evidence for similar phenomena in the action of alcohol, as shown later, is apparent in the changing slopes in a corresponding analysis with respect to the relation between inhibition and concentration of alcohol at different hydrostatic pressures. The formulation given in equation (10) for Type II inhibitors may be readily extended, as follows,

to include more than one equilibrium between the inhibitor and enzyme. Qualitatively a basis is thus provided for interpreting the phenomena observed, but it is quantitatively somewhat tedious to apply. In the equations below,  $K_1$  refers as usual to the normal reversible denaturation. The equilibrium between the enzyme and urethane,  $K_3$ , is now represented by two different equilibria,\* designated by a prime and double prime, respectively. Letting  $A_0$  represent the total luciferase, we may write the equation for luminescence intensity and proceed with the formulation as before.

$$I_1 = \frac{bk_2}{1 + K_1} (A_0)(LH_2) \quad (16)$$

$$(A_0) = (A_n) + (A_d) + (A_d U s') + (A_d U s'') \quad (17)$$

$$(A_0) = (A_n) + (A_n) K_1 + K_3' (U)^{s'} (A_n) + K_3'' (U)^{s''} (A_n) \quad (18)$$

$$(A_n) = \frac{(A_0)}{1 + K_1 + K_3' (U)^{s'} + K_3'' (U)^{s''}} \quad (19)$$

$$\frac{I_1}{I_2} = \frac{1 + K_1 + K_3' (U)^{s'} + K_3'' (U)^{s''}}{1 + K_1} \quad (20)$$

$$\left( \frac{I_1}{I_2} - 1 \right) = \frac{K_3'}{1 + K_1} (U)^{s'} + \frac{K_3''}{1 + K_1} (U)^{s''} \quad (21)$$

From equation (21) it is obvious that if the values of  $K_3'$  and  $K_3''$ , and of  $s'$  and  $s''$  are different from zero, the slopes of lines analyzed according to the simpler formulation for a single equilibrium will in general vary with either temperature or pressure. The net effect, of course, will be approximately indicated by the slopes of the lines obtained in the plot of  $\log \left( \frac{I_1}{I_2} - 1 \right)$  against log urethane concentration; *i.e.*, with increasing slope, the average ratio of drug-enzyme molecules increases. With rise in temperature the weaker of two equilibrium combinations would lose in importance as compared with the stronger. If the more exothermal  $K$  is associated with the larger  $s$ , the number combined will seem to rise with temperature as in Fig. 32.<sup>11</sup>

<sup>11</sup> In the literature are a number of examples in which the quantitative effects of urethane, studied at only one temperature and pressure on complex processes such as growth or respiration, have been analyzed in a manner corresponding to that used in the present instance, and have shown more or less pronounced departures from a straight line relation (*e.g.*, Fisher and Stern, 1942). Although there is an understandable tendency to interpret such results in terms of an effect of the drug on more than one distinct system, the data have generally been inconclusive on this point. When a drug is capable of combining with an enzyme at more than one site with different bond strengths, equation (21) is obtained. With appropriate values of the  $K$ 's and  $s$ 's the complications that have been observed are obtained.

The temperature analysis of the curves in Fig. 30 is given in Fig. 33, for the several concentrations of urethane. In contrast to the analysis of the sulfanilamide inhibition (Fig. 20), the lines in Fig. 33 are not largely straight throughout, but curve markedly towards greater inhibition in the region of the higher temperatures, much as in the analysis of luminescence intensity against temperature at alkaline pH. For reasons already indicated, this departure from the simplest straight line relation might be anticipated in

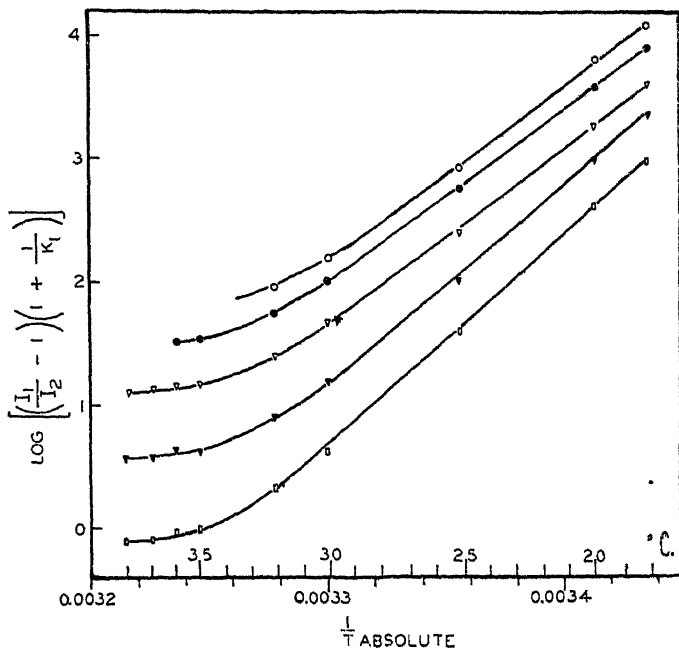


FIG. 33. Analysis of the data shown in Fig. 30 as a Type II inhibition, with respect to temperature, for the several concentrations of urethane.

connection with substances which cause an irreversible effect along with their reversible influence on the denaturation equilibrium, and in proportion to the time of exposure during which the rate process of the destruction is allowed to proceed. The values of  $\log \left[ \left( \frac{I_1}{I_2} - 1 \right) \left( 1 + \frac{I}{K} \right) \right]$  thus tend to be too high at the low values of  $1/T$ , and a bend occurs in the curve.

The straight line portions of the curves in Fig. 33 enable an estimate of the heat of reaction of the urethane-enzyme equilibrium. The average value amounts to about 65,000 calories, which is close to 60,300 calories found for the  $\Delta H$  in the denaturation equilibrium at high concentrations of hydroxyl ions.

(c) *Shift in "Optimum Temperature"*

In several instances considered thus far the inhibitor has caused an obvious change in the temperature at which the maximum luminescence intensity is observed. Thus, the normal optimum is shifted to slightly higher temperatures in the presence of inhibiting concentrations of hydrogen ions (Fig. 12) or of sulfanilamide (Figs. 17 and 18), while it is altered to lower temperatures in the presence of unfavorable concentrations of hydroxyl ions (Fig. 15) or of urethane (Figs. 30 and 31). The apparent activation energy for luminescence is increased by the former two, and decreased by the latter two agents, in the manner that has already been discussed. The apparent heat of reaction in the denaturation equilibrium, which at best can only be approximated, is likewise affected, to a greater extent by the urethane than the sulfanilamide type. Complicating factors that enter into the control of the over-all, observed rate of luminescence or other processes in living cells, make it impossible to arrive at very exact values for the activation energy, denaturation equilibrium, and other thermodynamic or rate constants of reactions that influence the process in question.

From the theory that we have already described, it follows that the optimum temperature will shift in the manner that is qualitatively apparent in the figures. Formulations may be readily derived for predicting quantitatively the amount of this shift, but the accuracy of the predictions is extremely sensitive to the experimental error as well as to any complicating influences within the cell, such as an irreversible effect in addition to the reversible equilibrium established with a given enzyme by an inhibitor, or an effect of the inhibitor on more than one system. With urethane, better than with sulfanilamide the constants that fit the curves predict with some accuracy the amount of the change in the optimum temperature, showing that in some cases the possible complicating factors are not of overshadowing significance, and results are in fair agreement with the simple theory. The theoretically predicted changes in optimum temperature, indicated by the arrows in Fig. 30, were arrived at through the following equations.

$$I = \frac{bk_2(LH_2)(A_0)}{1 + K_1 + K_1K_3U^*} \quad (22)$$

$$\ln I = \ln(b)(LH_2)(A_0) + \ln k_2 - \ln(1 + K_1 + K_1K_3U^*) \quad (23)$$

At the maximum, the change in luminescence intensity with respect to temperature is zero. Hence, at constant pressure,

$$0 = \frac{\partial \ln I}{\partial T} = \frac{1}{T} + \frac{\Delta H_2^\ddagger}{RT^2} - \frac{\frac{\Delta H_1}{RT^2} K_1 + \frac{\Delta H_1 + \Delta H_3}{RT^2} K_1 K_3 U^*}{1 + K_1 + K_1 K_3 U^*} \quad (24)$$

Multiplying through by  $RT^2 (1 + K_1 + K_1 K_3 U^s)$ , and dividing by  $RT + \Delta H_2^\ddagger = \Delta H_2'$ , we obtain:

$$1 + K_1 + K_1 K_3 U^s = \frac{\Delta H_1}{\Delta H_2'} K_1 + \frac{\Delta H_1 + \Delta H_3}{\Delta H_2'} (K_1 K_3 U^s) \quad (25)$$

$$1 + \left(1 - \frac{\Delta H_1}{\Delta H_2'}\right) K_1 = \left(\frac{\Delta H_1 + \Delta H_3}{\Delta H_2'} - 1\right) K_1 K_3 U^s \quad (26)$$

The change in optimum temperature is obtained by plotting  $y$  and  $z$  against temperature where

$$y = 1 + \left(1 - \frac{\Delta H_1}{\Delta H_2'}\right) K_1 \quad (27)$$

and

$$z = \left(\frac{\Delta H_1 + \Delta H_3}{\Delta H_2'} - 1\right) K_1 K_3 U^s \quad (28)$$

as shown in Fig. 34.

The temperature at which luminescence reaches a maximum intensity in different concentrations of  $U$  occurs at the intersection of  $y$  and  $z$ .

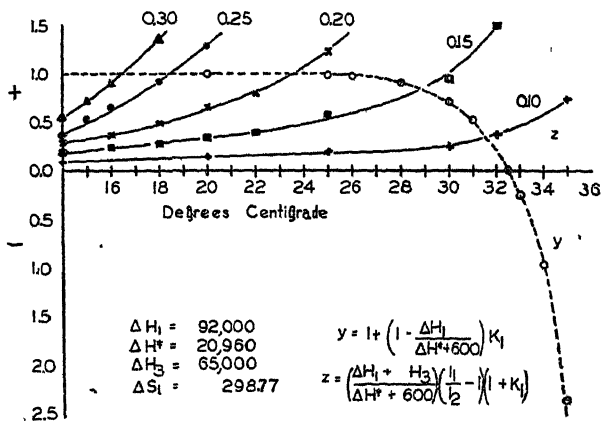


FIG. 34. Equations and data used in calculating the shifts in optimum temperature shown in Fig. 30.

#### VIII. THE ACTION OF ALCOHOL

In a previous publication (Johnson, Eyring, and Kearns, 1943) it was shown that, at low temperatures, the primary aliphatic alcohols containing from one to five carbon atoms appear to affect bacterial luminescence in a similar manner, though with different potencies. On plotting the logarithm

of  $\left(\frac{I_1}{I_2} - 1\right)$  against the logarithm of alcohol concentration, a series of practically parallel lines resulted, with the intercept on the abscissa generally at lower concentrations the longer the carbon chain of the alcohol. The slope of these lines indicated that a ratio of between two and three molecules of alcohol per enzyme molecule is formed in the equilibrium. Furthermore, at 22° C. the inhibition by a given concentration was greater than at 5° C.

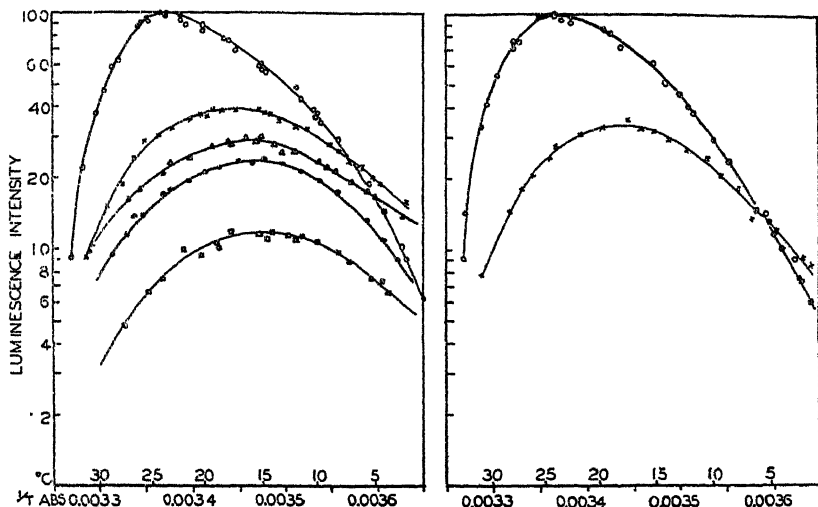


FIG. 35. The intensity-temperature relationship of luminescence in corresponding portions of a suspension of *P. phosphoreum* containing various concentrations of alcohol. The two graphs are for repeated experiments carried out on different days. Note the "stimulation" of luminescence by low concentrations of alcohol at low temperatures. Semilogarithmic scale. Open circles, control; crosses, 0.4 M alcohol in figure at left, 0.5 M in figure at right; triangles, 0.5 M alcohol; solid circles, 0.6 M alcohol; squares, 0.8 M alcohol.

These earlier results suggested that the simple alcohols act in much the same manner as urethane. Like urethane, they were found to enter into a loose complex formation with sulfanilamide, forming a combination sufficiently strong that, under appropriate conditions of temperature and concentrations, a mutual antagonism of their physiological inhibitions was evident. In the present study further data have been obtained with respect to the significance of temperature, and especial attention has been given the effects of hydrostatic pressure, in relation to both temperature and concentration.

(a) *The Effect of Alcohol in Relation to Concentration and Temperature*

The relation between temperature and luminescence intensity for suspensions of cells containing various concentrations of alcohol is shown in Fig. 35.



It is apparent that these results are similar, in certain distinct respects, to those obtained with urethane. The inhibitor causes a decrease in apparent activation energy, a decrease in apparent energy of denaturation, and a shifting of the normal optimum to lower temperatures. A "stimulating" effect of slight concentrations of alcohol at the lower temperatures, sometimes encountered with urethane, is also apparent in Fig. 35. This latter phenomenon is qualitatively similar to the urethane antagonism of sulfanilamide, as investigated by Johnson, Eyring, and Kearns (1943). The interpretation

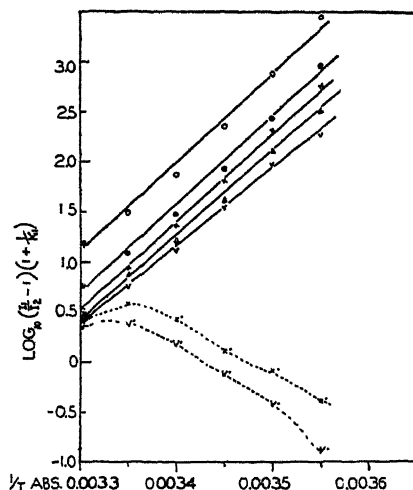


FIG. 36. Analysis of the data shown in Fig. 37 with respect to temperature. The solid lines represent  $\log_{10} \left[ \left( \frac{I_1}{I_2} - 1 \right) \left( 1 + \frac{I}{K_1} \right) \right]$  against  $1/T$ , appropriate to Type II inhibition, and the broken lines represent  $\log_{10} \left( \frac{I_1}{I_2} - 1 \right)$  against  $1/T$ , appropriate to Type I inhibition. The inhibition appears to conform to the former type.

is thus suggested that the stimulatory effects in the present instance take place through the combination of the alcohol with some naturally occurring metabolite which ordinarily exerts a slightly retarding influence on the overall rate of the luminescent reactions. The major effect, that of inhibition at the higher concentrations and temperatures, has been analyzed in the same manner as with urethane, and the results plotted in Fig. 36. The dotted lines show the results of attempting an analysis according to the formulations for the sulfanilamide type of luminescence inhibitor. The urethane type is the one to which the data conform, as evidenced by both the direction of slope and linearity of the lines. The fact that the lines are almost linear indicates that the effect of the alcohol is, for the most part, either on the pace setting reaction

or on a preceding reaction with similar temperature coefficient. The fact that they are not completely linear, but rather tend to curve in the direction of greater inhibition at the higher temperatures, shows that complicating effects, such as those discussed earlier, and also as evidenced by a stimulatory action at low temperature, are present in this case also. The slopes of the line, however, again permit an estimate of the heat of reaction in the alcohol equilibrium, amounting to approximately—37,000 calories. This value is considerably less than that for urethane, and for hydroxyl ions.

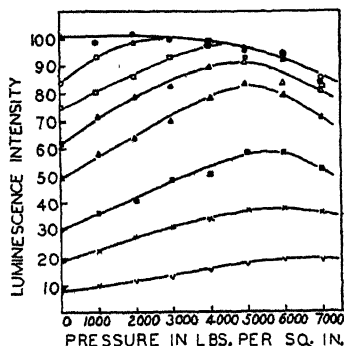


FIG. 37. Luminescence intensity of *P. phosphoreum* as a function of alcohol concentration and hydrostatic pressure. The intensity of the control, without alcohol and at normal pressure, is arbitrarily taken as equal to one hundred. Note the complete elimination of the apparent inhibition of alcohol in low concentrations by hydrostatic pressure. Note, also, the shift in observed optimum pressure with increasing concentrations of alcohol. The concentrations of alcohol, represented by successive curves, from top to bottom of the figure are as follows: 0 (control), 0.2, 0.4, 0.5, 0.6, 0.8, 1.0, and 1.5 molar. The temperature was 17.5° C. throughout.

#### (b) Inhibition in Relation to Concentration and Pressure

In studying the relation between pressure and inhibition of luminescence by different concentrations of alcohol at a given constant temperature, advantage may be taken of the fact that, in the neighborhood of the normal optimum temperature, pressure has very little effect on the observed intensity of luminescence, except when certain inhibitors are present. In Fig. 37 the relation between pressure, concentration of alcohol, and luminescence intensity is shown with respect to the control without alcohol, and at atmospheric pressure, arbitrarily taken as 100. The pressure effects were readily reversible, on release of pressure.

Noteworthy among the things that are qualitatively apparent in Fig. 37 are the following. In the first place, it will be noted that in the absence of added alcohol, pressure alone causes only a slight diminution in luminescence, beginning at about 4,000 pounds per square inch, and increasing to about 14

per cent at 7,000 pounds. With an initial addition of alcohol, however, resulting in a final concentration of 0.2 M, luminescence is reduced to around 16 per cent, but this inhibition becomes less on application of hydrostatic pressure, and disappears at about 2,000 pounds. With increasing concentrations of alcohol pressure again tends to counteract the inhibition, but if the alcohol concentration is higher than about 0.4 M, the inhibition is not completely abolished, for the high-pressure diminution of luminescence, observed in the absence of alcohol, now becomes more significant than the effect of pressure in eliminating the alcohol inhibition. Thus, with increasing concentrations of alcohol there is a very pronounced shift in the pressure at which maximum luminescence is observed.

The data in Fig. 37 include two variables, concentration of alcohol and hydrostatic pressure, other factors such as temperature, pH, etc., remaining constant. The general formulations which have been derived may be applied in an analysis from the point of view of either of these variables. An analysis of the relation between inhibition and concentration, for different pressures, is shown in Fig. 38.

In agreement with the earlier study that was carried out at normal pressure, Fig. 38 shows that a fairly steep slope of 2.8 is obtained. At higher pressures the slope becomes greater, amounting to 3.1 at 3,000 pounds, 3.8 at 4,000 pounds, and 4.0 at 7,000 pounds. It is difficult to see how this change in slope could represent an increase in the ratio of alcohol to enzyme molecules if only a single equilibrium between the two were involved. On the basis that there is more than one equilibrium between the two molecules, however, the phenomenon may be readily accounted for, in the manner discussed for the action of urethane at different temperatures.

In analyzing the data of Fig. 37 from the point of view alternative to the above, *viz.* with reference to the relation between the effects of constant concentrations of alcohol and different hydrostatic pressures, it becomes necessary to calculate the appropriate values of  $K_1$  for each pressure. These values may be obtained from the relation:

$$\Delta F_{p_2}^0 = \Delta F_{p_1}^0 + \int_{p_1}^{p_2} \overline{\Delta V} dp \quad (29)$$

At the temperature of 17.8°C., used in this experiment, the value of  $K_1$  varies between 0.03404 and 0.0295, or only about 1.15 times, throughout the range between normal pressure and 7,000 pounds. The results of the analysis are shown in Fig. 39.

There is a pronounced change in slope of the curves in Fig. 39 with increasing concentrations of alcohol, and a tendency to depart from a straight line relation that is very apparent at the higher pressures. It is evident, therefore, that the action of alcohol involves more than one equilibrium. Complications

such as those already discussed in connection with the temperature-concentration relation of the urethane inhibition, and the action of pressure on the rate of irreversible denaturation are evidently concerned in this case also.

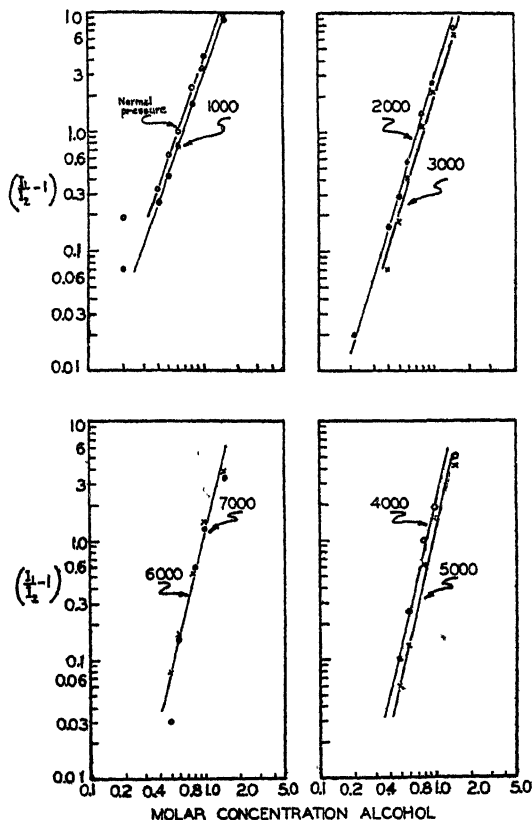


FIG. 38. Analysis of the data shown in Fig. 37 concerning the relation between inhibition and alcohol concentration at different given hydrostatic pressures. Note the increase in the slope at the lines from about 2.8 at normal pressure to 4.0 at 7,000 pounds per square inch. Log-log scale.

Moreover, the fact that a stimulatory effect of alcohol is observed at low temperatures and concentrations shows that there are additional reactions which necessitate an extension of the theory. The data in Fig. 39, while not permitting an exact analysis of the molecular volume changes involved in the effects of alcohol on the luminescent system, are useful in showing that the volume changes are large. In addition, the agreement of the analysis with the simple theory is sufficiently good to indicate that the complicating reactions are of secondary importance. A somewhat clearer view of the chief mechanism

is obtained through a study of the effects of a given concentration of alcohol in relation to both temperature and hydrostatic pressure, as discussed below.

(c) *The Inhibition in Relation to Pressure and Temperature*

The pressure effect on luminescence after addition of alcohol to give a final concentration of 0.5 molar is shown for four different temperatures in Fig. 40.<sup>12</sup>

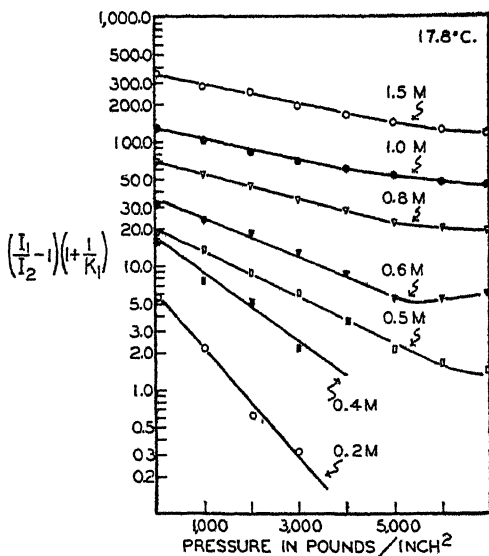


FIG. 39. Analysis of the data shown in Fig. 37 with respect to pressure and each of the given concentrations of alcohol.

In this figure the luminescence of cells without added alcohol and at normal pressure has again been set arbitrarily equal to 100, and the remaining points multiplied by the appropriate factor for this scale. It should be recalled that the intensity actually observed at normal pressure is very different at the several temperatures. The direction of the pressure effect, however, as well as its influence on the per cent inhibition, is most readily apparent in this plot. Thus, at temperatures below the normal optimum, such as 11.1° C., luminescence in the control continually diminishes as the pressure is raised, thereby opposing the volume increase of activation in the rate process. At this temperature the value of  $K_1$  is too small for pressure to counteract, through its influence on the denaturation equilibrium, the effect on the rate process. If alcohol is added, however, an equilibrium is established that is accompanied by a volume increase of the enzyme, and a decrease in catalytic activity. The

<sup>12</sup> This figure is remarkably like the one obtained in a similar study with regard to the action of quinine (Johnson and Schneyer, 1944).

net effect of pressure thus becomes altogether different. At 11° C., a concentration of 0.5 molar alcohol causes just enough reversible denaturation that the effect of increasing pressures in slowing the light-emitting reaction is counterbalanced by the effect of supplying more of the catalytically active enzyme, through the denaturation equilibrium. Consequently, in the alcohol-containing cells there appears to be no effect of pressure under these conditions.

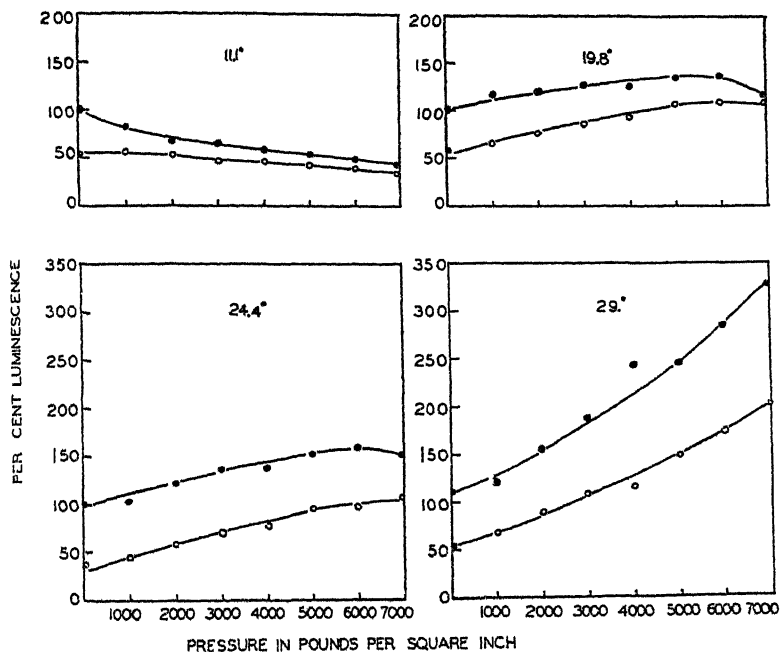


FIG. 40. The effect of pressure on the inhibition of luminescence by 0.5 molar alcohol at different temperatures. The intensity at normal pressure, without alcohol, has arbitrarily been taken as equal to one hundred at each temperature.

In comparison with the control, of course, the inhibition continues to decrease with rise in pressure, and the nearly horizontal line representing luminescence intensity of the alcohol suspension is approached by the continually falling line of the control. The same general mechanism is responsible for the net effects of pressure observed at the other temperatures, both in the control and the alcohol-containing suspensions; *i.e.*, in all cases the difference between the retarding and accelerating effects of the pressure respectively, on at least the two reactions. At temperatures considerably above the optimum, *e.g.* 29° C., at which the value of  $K_1$  is large and much of the enzyme is in the reversibly denatured condition, pressure causes a striking increase in the intensity of luminescence, both in the control and in the alcohol-containing suspension.

Since the actual intensity at this temperature, in comparison with the intensity at the normal optimum, has already been diminished by the above-optimum temperature, the several hundred per cent increase that occurs on application of pressure does not cause the luminescence intensity ever to exceed, to any considerable extent, the normal maximum.

An approximate analysis of the data in Fig. 40 with respect to the alcohol inhibition is shown in Fig. 41. A more precise treatment would involve calculating the appropriate values of  $K_1$  at each temperature as well as each

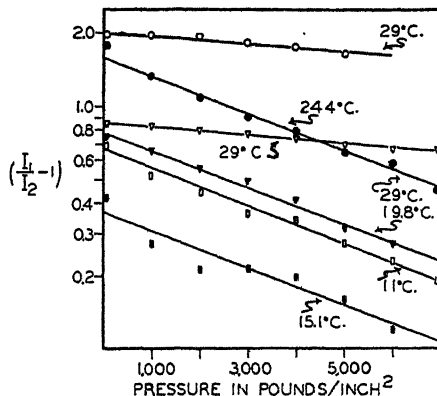


FIG. 41. Analysis of the data shown in Fig. 40 with regard to the effect of pressure on the alcohol inhibition at different temperatures. This analysis would be more precise if the values of  $\left(\frac{I_1}{I_2} - 1\right)$  on the ordinate were multiplied by  $\left(1 + \frac{1}{K_1}\right)$ . Although the slopes in general are not greatly altered, the line for 29° C. becomes practically horizontal when the values of  $K_1$  for the different pressures are taken into account. The two lines shown for 29° are from separate experiments.

pressure, and multiplying each point obtained for  $\left(\frac{I_1}{I_2} - 1\right)$  by the proper value of  $\left(1 + \frac{1}{K_1}\right)$ , as before. This procedure has been carried out only for the curve for 29°. This curve, already nearly horizontal with respect to the abscissa, becomes almost perfectly horizontal when the values of  $K_1$  are taken into account. Although we again have evidence of other reactions that enter into the measured results and thereby tend to complicate the picture that has been drawn on the assumption of the simplest conditions, the data appear adequate and sufficiently clear to justify a somewhat more detailed interpretation with regard to the chief mechanism, as follows: In the absence of alcohol, we have as usual

$$I_1 = \frac{bk_2(A_0)(\text{LH}_2)}{1 + K_1} \quad (30)$$

When alcohol in molar concentration ( $U$ ) is added, this becomes

$$I_2 = \frac{bk_2}{1 + K_1 + (U)^s K_3 K_1} \quad (31)$$

Dividing equation (30) by equation (31),

$$\frac{I_1}{I_2} = 1 + \frac{K_3 K_1 (U)^s}{1 + K_1} \quad (32)$$

$$\left(\frac{I_1}{I_2} - 1\right) \left(1 + \frac{1}{K_1}\right) = K_3 (U)^s \quad (33)$$

Letting the superscript (0) represent  $K$  at normal pressure,

$$K_1 = K_1^0 e^{-p\Delta V_1} \quad \text{and} \quad K_3 = K_3^0 e^{-p\Delta V_3}, \quad (34)$$

Substituting these expressions for  $K$ 's, and taking the logarithm,

$$\ln \left(\frac{I_1}{I_2} - 1\right) = \ln \left(\frac{K_3 K_1}{1 + K_1}\right) + s \ln U = \ln \left(\frac{K_3^0 K_1^0}{1 + K_1^0 e^{-p\Delta V_1}}\right) + \left(\frac{-\Delta V_3 - \Delta V_1}{RT}\right) p + s \ln U \quad (35)$$

$$\ln \left(\frac{I_1}{I_2} - 1\right) = \ln K_3^0 K_1^0 - \ln (1 + K_1^0 e^{-p\Delta V_1/RT}) + \left(\frac{-\Delta V_3 - \Delta V_1}{RT}\right) p + s \ln U \quad (36)$$

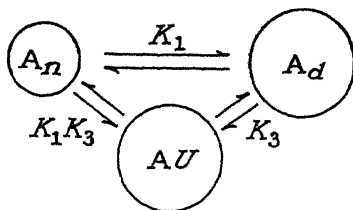
From equation (35), it is apparent that at temperatures where  $K_1$  is small in comparison with 1, then the  $\ln \left(\frac{I_1}{I_2} - 1\right)$  gives the approximate value of  $\ln (K_3 K_1)$  plus  $s \ln U$ . The expression  $s \ln U$  remains constant for a given concentration of alcohol. As the value of  $K_1$  becomes large in comparison with 1, then  $\left(\frac{K_1}{1 + K_1}\right)$  tends to cancel out, and the  $\ln \left(\frac{I_1}{I_2} - 1\right)$  gives the approximate value of  $\ln K_3 + s \ln U$ . Thus at relatively low temperatures, such as 11.1° and 19.8° C., where the value of  $K_1$  is small, the slopes of the lines in Fig. 41 give the sum of the volume changes of the normal denaturation equilibrium,  $K_1$ , and the equilibrium  $K_3$  characterizing the reaction between the alcohol and the protein. At the relatively high temperatures, on the other hand, the slope represents the volume change almost wholly with respect to  $K_3$ .

Since in Fig. 41 the slope of the line at 29° is so nearly horizontal, it follows that the value of  $\Delta V_3$  must be extremely small in comparison with  $\Delta V_1$ . In other words, the volume change responsible for the observed pressure effects on the alcohol inhibition is confined practically altogether to the changes which this drug causes in the normal denaturation equilibrium,  $K_1$ . Thus, the volume change indicated by the slopes of the lines for the lower temperatures, in Fig. 41, should yield values for  $\Delta V$  that correspond to the values obtained from the effects of pressure, at higher temperatures, on suspensions to which no alcohol



has been added. An exact comparison of the values obtained in the two cases is hardly possible, inasmuch as the temperature dependence of  $\Delta V_1$ , pointed out in the study of Eyring and Magee (1942), as well as other facts, show that the situation is more complicated than the theory takes into account. However, the value of  $\Delta V_1$  at 35° C., according to the results of Eyring and Magee, is 64.6 cc. The data from the alcohol inhibition, in Fig. 41, as based on the slope of the curve at 24° C., which is practically parallel to the others at the lower temperatures, indicate a  $\Delta V_1$  of approximately 62.6 cc. Although the closeness of this striking agreement is no doubt partly fortuitous, it is in line with the theoretical expectations.

Diagrammatically, we might picture in simplest terms the equilibria primarily concerned in the relation of temperature and pressure to the inhibitory action of alcohol as follows:—



There is no physical way of distinguishing whether the alcohol-enzyme compound is formed as a result of a combination of this drug with the reversibly denatured form of the enzyme, according to the equilibrium constant,  $K_3$ , or with the native form of the enzyme, according to equilibrium constant  $K_3$  times equilibrium constant  $K_1$ . The volume change and end result are the same in either case.

With regard to the mechanism by which alcohol combines with the protein and promotes the reversible denaturation, the general similarity between the observed effects of alcohol and urethane suggests that the action is fundamentally the same in the two cases.

#### IX. DISCUSSION

The significance of this study has several interrelated aspects, which are perhaps worth considering very briefly from the points of view of (1), the phenomenon of bioluminescence in particular, (2), the mechanism and control of biological oxidative reactions in the living cell and in extracts, and (3), the physical chemistry of inhibitions caused by drugs and other factors that act upon various enzyme systems.

With regard to the problem of luminescence, the results obtained in experiments with the various factors that influence the rate, or intensity, of this process in bacteria have made it possible to schematize, in somewhat greater

detail and clarity than previously, certain of the fundamental reactions concerned. The hypotheses advanced take into account the previously reported data derived from studies of kinetics, as well as the chemical and physical properties of the system, and provide a kinetic basis for interpreting several more or less obscure phenomena; *e.g.*, the relation between the luminescent and non-luminescent oxidation of luciferin. The final identity of the molecules that participate in the light-emitting system, and their rôle in cellular oxidations remain to be established, although the theoretical chemistry of the reactions has been examined in some detail, and has been brought to a stage satisfactory for an analysis of rate-controlling influences, as well as for a partial understanding of the fundamental processes involved.

In undertaking to elucidate more clearly the luminescent oxidation itself, it has been necessary to consider the action of various factors, such as hydrogen ion concentration, temperature, and pressure, both separately and in relation to each other. Some of the phenomena observed have been typical of those familiarly encountered in the study of these factors in relation to diverse biological processes. Presumably the basic mechanisms involved in many other cases will be the same as in this one. Some new relationships, such as that between the pH and the effect of pressure on the reaction velocity of an enzyme system, have been studied for the first time. The results have contributed to a somewhat more complete picture of events in the catalytic system during the process of electron transfer, and of the intimate mechanism involved in specific inhibitions and activations of such systems; *e.g.*, by sulfanilamide and *p*-aminobenzoic acid, respectively.

Finally, in investigating and accounting for the action of various drugs, etc., which influence the rate of the over-all process of luminescence, new formulations have been derived appropriate to the phenomena in question. These formulations are of general applicability for the purpose of testing the conformity of data from experiments to certain basic mechanisms. In luminescence, most of the observed effects appear to involve one chief site, and there are indications that this site is the luciferase molecule itself, in which the luciferin possibly occupies the rôle of prosthetic group to the protein enzyme. The theory accounts for two distinct types of inhibitions, in relation to temperature, pressure, hydrogen ion concentration, and constancy with time: Type I, caused by substances which combine with, or in place of, the prosthetic group, independently of the reversible protein denaturation; and Type II, caused by drugs which combine in a manner that promotes the reversible denaturation of the protein. In the latter type an irreversible reaction usually takes place along with the reversible change. It is of particular interest to note that, in this latter group, are a number of "lipoid-soluble narcotics" whose fundamental mode of action has long remained obscure. There is increasing evidence, however, to indicate that the effects of these substances, even in complex

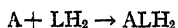
phenomena such as narcosis of nerves, are mediated through oxidative enzyme systems (Quastel, 1943), apparently not very different from the luminescent system. The theory outlined in the present study, therefore, should be of use in this connection also.

In the literature are numerous examples of phenomena qualitatively similar, in one or another respect, to those described in this paper. Unfortunately, the quantitative data are generally insufficient for an analysis on the basis of the present theory. This is generally true of the data with respect to temperature, and almost wholly true with regard to pressure. Further and more complete studies with adequate reference to the several variables of pH, temperature, pressure, and concentration of specific inhibitors or activators, are needed in order to understand the theoretical chemistry concerned. In so far as studies of this kind contribute to the solution of biological and related problems they may be considered eminently worth while. The theory advanced herein will perhaps aid the progress of such investigations.

The authors wish to acknowledge, with much appreciation, the cooperation of Dr. E. N. Harvey in providing much of the apparatus used for measuring luminescence, and for studying hydrostatic pressure effects. Indebtedness is also gratefully acknowledged to Dr. L. Michaelis for the special compounds tested for a possible identity with luciferin. The pressure bomb was gold-plated through the kindness of Dr. Thon. Valuable assistance in the pressure experiments was rendered by Dr. Leon Schneyer, and in some of the other experiments by Miss Nina Zworykin. Indebtedness is also acknowledged for valuable discussions with Dr. Harvey, Dr. Chase, and Dr. McElroy.

#### X. SUMMARY

On the basis of available data with regard to the chemical and physical properties of the "substrate" luciferin ( $\text{LH}_2$ ) and enzyme, luciferase (A), and of kinetic data derived both from the reaction in extracts of *Cypridina*, and from the luminescence of intact bacteria, the fundamental reactions involved in the phenomenon of bioluminescence have been schematized. These reactions provide a satisfactory basis for interpreting the known characteristics of the system, as well as the theoretical chemistry with regard to the control of its over-all velocity in relation to various factors. These factors, here studied experimentally wholly with bacteria, *Photobacterium phosphoreum* in particular, include pH, temperature, pressure, and the drugs sulfanilamide, urethane, and alcohol, separately and in relation to each other. Under steady state conditions of bacterial luminescence, with excess of oxidizable substrate and with oxygen not limiting, the data indicate that the chief effects of these agents center around the pace setting reactions, which may be designated by the equation:



following which light emission is assumed proportional to the amount of the excited molecule,  $AL^*$ .

The relation between pH and luminescence intensity varies with (a), the buffer mixture and concentration, (b), the temperature, and (c), the hydrostatic pressure.

At an optimum temperature for luminescence of about 22° C. in *P. phosphoreum*, the effects of increasing or decreasing the hydrogen ion concentration are largely reversible over the range between pH 3.6 and pH 8.8. The relation between luminescence intensity and pH, under the experimental conditions employed, is given by the following equation, in which  $I_1$  represents the maximum intensity, occurring about pH 6.5;  $I_2$  the intensity at any other given pH;  $K_5$  the equilibrium constant between hydrogen ions and the  $AL^-$ ; and  $K_6$  the corresponding constant with respect to hydroxyl ions:

$$\left(\frac{I_1}{I_2} - 1\right) = K_5 (H^+) + K_6 (OH^-)$$

The value of  $K_6$ , as indicated by the data, amounts to  $4.84 \times 10^4$ , while that of  $K_5$  amounts to  $4.8 \times 10^5$ .

Beyond the range between approximately pH 3.8 and 8.8, destructive effects of the hydrogen and hydroxyl ions, respectively, were increasingly apparent. By raising the temperature above the optimum, the destructive effects were apparent at all pH, and the intensity of the luminescence diminished logarithmically with time. With respect to pH, the rate of destruction of the light-emitting system at temperatures above the optimum was slowest between pH 6.5 and 7.0, and increased rapidly with more acid or more alkaline reactions of the medium.

The reversible effects of slightly acid pH vary with the temperature in the manner of an inhibitor (Type I) that acts independently of the normal, reversible denaturation equilibrium ( $K_1$ ) of the enzyme. The per cent inhibition caused by a given acid pH in relation to the luminescence intensity at optimum pH, is much greater at low temperatures, and decreases as the temperature is raised towards the optimum temperature. The observed maximum intensity of luminescence is thus shifted to slightly higher temperatures by increase in ( $H^+$ ).

The apparent activation energy of luminescence is increased by a decrease in pH. The value of  $\Delta H^\ddagger$  at pH 5.05 was calculated to be 40,900 calories, in comparison with 20,700 at a pH of 6.92. The difference of 20,200 is taken to represent an estimate of the heat of ionization of  $ALH$  in the activation process, and compares roughly with the 14,000 calories estimated for the same process, by analyzing the data from the point of view of hydrogen ions as an inhibitor. The decreasing temperature coefficient for luminescence in proceeding from low temperatures towards the optimum is accounted for in part by the greater degree of ionization of  $ALH$ .

At the optimum temperature and acid reactions, pressures up to about 500 atmospheres retard the velocity of the luminescent oxidation. At the same temperature, with decrease in hydrogen ion concentration, the pressure effect is much less, indicating a considerable volume increase in the process of ionization and activation.

In the extremely alkaline range, beyond pH 9, luminescence is greatly reduced, as compared with the intensity at neutrality, and under these conditions pressure causes a pronounced increase in intensity, presumably by acting upon the reversible denaturation equilibrium of the protein enzyme, A.

Sulfanilamide, in neutral solutions, acts on luminescence in a manner very much resembling that of hydrogen ions at acidities between pH 4.0 and pH 6.5. Like the hydrogen ion equilibrium, the sulfanilamide equilibrium involves a ratio of approximately one inhibitor molecule to one enzyme molecule. The heat of reaction amounts to about 11,600 calories or more in a reversible combination that evidently evolves heat. Like the action of H ions, sulfanilamide causes a slight shifting of maximum luminescence intensity in the direction of higher temperatures, and an increase in the energy of activation.

The effect of sulfanilamide on the growth of broth cultures of eight species of luminous bacteria indicates that there is no regular relationship among the different organisms between the concentration of the drug that prevents growth, and that which prevents luminescence in the cells which develop in the presence of sulfanilamide. *p*-Aminobenzoic acid (PAB) antagonizes the sulfanilamide inhibition of growth in luminous bacteria, and the cultures that develop are luminous. When (PAB) is added to cells from fully developed cultures, it has no effect on luminescence, or causes a slight inhibition, depending on the concentration. With luminescence partly inhibited by sulfanilamide, the addition of PAB has no effect, or has an inhibitory effect which adds to that caused by sulfanilamide. Two different, though possibly related, enzyme systems thus appear to limit growth and luminescence, respectively. The possible mechanism through which both the inhibitions and the antagonism take place is discussed.

The irreversible destruction of the luminescent system at temperatures above that of the maximum luminescence, in a medium of favorable pH to which no inhibitors have been added, proceeds logarithmically with time at both normal and increased hydrostatic pressures. Pressure retards the rate of the destruction, and the analysis of the data indicates that a volume increase of roughly 71 cc. per gm. molecule at 32° C. takes place in going from the normal to the activated state in this reaction. At normal pressure, the rate of destruction has a temperature coefficient of approximately 90,000 calories, or about 20,000 calories more than the heat of reaction in the reversible denaturation equilibrium. The data indicate that the equilibrium and the rate process are

two distinct reactions. The equation for luminescence intensity, taking into account both the reversible and irreversible phases of the reaction is given below. In the equation  $b$  is a proportionality constant;  $k'$  the rate constant of the luminescent reaction;  $A_0$  the total luciferase;  $A_{0i}$  the total initial luciferase at time  $t$  equals 0;  $k_n$  the rate constant for the destruction of the native, active form of the enzyme;  $k_d$  the rate constant for the destruction of the reversibly denatured, inactive form;  $t$  the time; and the other symbols are as indicated above:

$$I = \frac{bk'(LH)(A_0)}{1 + K_1} = \frac{bk'(LH)(A_{0i})}{1 + K_1} e^{-(k_n + k_d K_1 / 1 + K_1) t}$$

For reasons cited in the text,  $k_n$  evidently equals  $k_d$ .

Urethane and alcohol, respectively, act in a manner (Type II) that promotes the breaking of the type of bonds broken in both the reversible and irreversible reactions and so promotes the irreversible denaturation. This result is in contrast to the effects of sulfanilamide, which at appropriate concentrations may give rise to the same initial inhibition as that caused by urethane, but remains constant with time.

The inhibition caused by urethane and alcohol, respectively, increases as the temperature is raised. As a result, the apparent optimum is shifted to lower temperatures, and the activation energy for the over-all process of luminescence diminishes. An analysis for the approximate heat of reaction in the equilibrium between these drugs and the enzyme, indicates 65,000 calories for urethane, and 37,000 for alcohol. A similar analysis with respect to the effect of hydroxyl ions as the inhibitor gives 60,300 calories.

The effects of alcohol and urethane are sensitive to hydrostatic pressure. Moderate inhibitions at optimum temperature and pH, caused by relatively small concentrations of either drug, are completely abolished by pressures of 3,000 to 4,000 pounds per square inch.

At optimum temperature and pH, increasing concentrations of alcohol caused the apparent optimum pressure for luminescence to shift markedly in the direction of higher pressures. Analysis of the data with respect to concentration of alcohol at different pressures indicated that the ratio of alcohol to enzyme molecules amounted to approximately 4, at 7,000 pounds, but only about 2.8 at normal pressures. This phenomenon was taken to indicate that more than one equilibrium is established between the alcohol and the protein. A similar interpretation was suggested in connection with the fact that analysis of the relation between concentration of urethane and amount of inhibition at different temperatures also indicated a ratio of urethane to enzyme molecules that increased with temperature in the equilibria involved.

Analysis of the data with respect to pressure and the inhibition caused by a given concentration of alcohol at different temperatures indicated that the

volume change involved in the combination of alcohol with the enzyme must be very small, while the actual effect of pressure is apparently mediated through the reversible denaturation of the protein enzyme, which is promoted by alcohol, urethane, and drugs of similar type.

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# THE TEMPERATURE COEFFICIENT OF THE UREA DENATURATION OF EGG ALBUMIN

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When urea acts on a protein the progress of the reaction depends upon the concentration of protein, the concentration of urea, the pH, and the temperature of the solution. In the change spoken of as urea denaturation, there are at least four reactions involved: (1) the formation of a urea-protein complex; (2) the denaturation of the molecule with change in its typical biological properties; (3) the change in solubility of the molecule, which is often spoken of as denaturation; (4) the splitting of the molecule (at high concentrations of urea only).

Unfortunately, it is not easy or even possible to separate these four reactions. Although the swelling of proteins in urea solutions, freezing point determinations of proteins in urea, and x-ray patterns have given definite evidence of the existence of a urea-protein complex, there is no work so far reported that measures the rate of reaction 1, and it is generally assumed that it takes place so rapidly, especially at high concentrations of urea, that it does not affect the rate of the other three reactions. It is a preliminary reaction and if not followed by subsequent changes in the protein molecule, is probably reversible. It may, however, be a factor in the slow rate of denaturation of egg albumin with urea concentrations under 20 per cent (1) and may determine the critical concentration below which urea produces no inactivation in viruses (2).

In the case of hemoglobin, it has been shown (3) that reaction 4 can proceed without any effect on the specific biological properties of the molecule; *i.e.*, without reaction 2 taking place.

In the case of the effect of urea on tobacco mosaic virus, which has been exhaustively studied by Lauffer and Stanley (4-6), the methods used were measurements of the rate of reaction 4, the splitting of the molecule. Only one method in which the urea-protein mixture was diluted so as to precipitate the urea-denatured virus measured reaction 3, the rate of change of solubility of the protein. As Lauffer finds that the rate of change of solubility runs parallel with the rate of splitting of the molecule, except in the first few minutes of the reaction, he concludes that the split protein is the protein which shows loss of solubility in dilute solution. He does, however, state that the virus may lose its infectivity before the protein disintegrates. In other words, reaction 2 may precede reactions 3 and 4.

The picture in the case of egg albumin is very different. There is no method for measuring reactions 1 and 2, and the progress of denaturation is usually followed by measuring the rate of reaction 3, the loss of solubility in dilute solutions. This proceeds at urea concentrations too low to split the molecule (1). When the concentration of urea is high enough to split the molecule, it is found in the experiments reported here that the split molecule is soluble after the urea has been dialyzed out. Consequently, at high concentrations of urea where both reaction 3 and reaction 4 take place, the rate of formation of insoluble protein, which is the usual measurement of denaturation, is decreased in proportion to the rate of progress of reaction 4, the splitting of the protein.

In comparing denaturation changes in different proteins, it is important to analyze the reactions involved and compare results on the same reactions. In the case of tobacco mosaic virus, the split denatured protein is insoluble in dilute solutions so that measurements of reactions 3 and 4 run parallel. In the case of egg albumin, although denaturation makes the molecule insoluble in dilute solutions, splitting of the molecule makes the denatured egg albumin more soluble, so that the faster reaction 4 proceeds the less apparent denaturation there is, if denaturation is measured by the amount of insoluble material formed. It is not possible to tell whether reaction 3 must precede reaction 4, or whether the two reactions proceed independently and simultaneously. The results reported in this paper tend to support the view that denaturation (reaction 3) precedes splitting of the molecule (reaction 4).

Hopkins (7) studied the temperature coefficient of the denaturation of 5 per cent egg albumin solutions at pH 6.0 in the presence of 60 per cent urea. He found that denaturation, as measured by the insoluble protein formed on dilution, progressed more rapidly at 0° than at room temperature. Lauffer (6), studying the rate of splitting of tobacco mosaic virus with 6 M urea found that the specific reaction velocity was a minimum at room temperature and increased at higher or lower temperatures. He suggests that this U-shaped temperature coefficient curve may be characteristic of the denaturation of proteins, and that with changes in the concentration of urea the bottom of the U may shift, giving a positive temperature coefficient in the 0–40°C. range for low concentrations of urea, and a negative temperature coefficient at high concentrations. This U-shaped curve, he believes, is due to the fact that there are several simultaneous reactions going on, some with a positive and one at least with a negative temperature coefficient.

In the case of egg albumin, where splitting makes the denatured protein soluble, the results of the experiments reported here with different concentrations of urea show that the negative temperature coefficient reported by Hopkins is probably due to the fact that both reactions, denaturation (reaction 3) and splitting (reaction 4), have positive temperature coefficients. If reaction 4 has a higher temperature coefficient than reaction 3, it will decrease the

amount of insoluble protein formed as the temperature increases and therefore it will decrease the apparent rate of denaturation. This is actually the interpretation given his results by Hopkins who says that the figures present the "simulacrum" of a negative coefficient, and that he believes this to be due to the fact that "dissociation increases with increase in temperature sufficiently to account for the observed diminution in rate of denaturation." This is very different from saying that the urea denaturation of egg albumin has a negative temperature coefficient, which has been the usual interpretation given his results by other investigators. During the period of observation (3 hours) he found no diminution in the amount of insoluble protein, but he states also that if the urea-protein mixtures stand several days they remain clear on dialysis, which is obviously due to the splitting of all the denatured protein, and an apparent reversal of the denaturation process when that is measured by the amount of insoluble protein formed.

The following experiments show clearly that both denaturation (reaction 3) and splitting (reaction 4) have positive temperature coefficients, but at high concentrations of urea, where splitting becomes rapid, the increase in rate of splitting with increase in temperature will result in an apparent diminution in the rate of denaturation, when this is measured by the amount of insoluble protein formed on dilution or dialysis. Consequently, experiments performed with low concentrations of urea (less than 35 per cent) will show a positive temperature coefficient (1), as the amount of splitting has not been great enough to decrease the amount of insoluble protein formed. And experiments performed with 50 to 60 per cent urea, where splitting takes place rapidly, will give the appearance of a negative temperature coefficient (7).

### *Method*

The material used was isoelectric egg albumin (0.6 per cent before addition of urea) prepared by the method given in previous publications (8, 9). On addition of urea there was a shift in the pH value to pH 5.4.

Measurements of the opalescence of the solution were made by means of the Tyndall meter and Macbeth illuminometer, as previously described (9). The opalescence of the Tyndall beam, which serves as a measure of the amount of insoluble material present, is given in apparent foot-candles.

Results are given for egg albumin solutions to which urea had been added in concentrations of 40 and 50 per cent. These urea-albumin solutions were kept at 13°, 25°, and 40°C., for varying periods of time. They were then dialyzed against distilled water to remove the urea. This resulted in precipitation of the egg albumin molecules, in which reaction 3 had taken place, and in which reaction 4 had not taken place. The amount of insoluble material was measured by the opalescence of the Tyndall beam and is a measure of the amount of reaction 3, minus the amount of reaction 4 that had taken place.



## RESULTS

*Denaturation with 50 Per Cent Urea.*—In Table I the results found with 50 per cent urea are given for solutions kept at 13°, 25°, and 40°C. for varying periods of time. These figures are average values found from a series of observations at each temperature and give the amount of insoluble material present as determined by the opalescence of the Tyndall beam in apparent foot-candles. Part of these results are shown also in Fig. 1.

From the results given in Table I and Fig. 1, it is obvious that any calculation of temperature coefficients from the amount of insoluble material formed is meaningless when the urea concentration is high enough to result in rapid split-

TABLE I

Time	Amount of insoluble protein measured by Tyndall beam readings in apparent foot-candles		
	40°C.	25°C.	13°C.
10 min.	2.7		
15 "	3.0	3.5	
20 "	2.7		
30 "	2.2	4.6	3.7
45 "	2.1		
50 "	1.83		
1 hr.		5.5	5.2
1.5 "	1.35	5.3	6.0
2 "	1.17	4.8	5.4
2.5 "			5.2
3 "		4.5	4.8
5 "			3.6
11 "		3.2	
24 "		2.25	3.3
55 "		1.82	
69 "			2.75

ting of the denatured molecule, as the opalescence is the result of two antagonistic reactions both with a positive temperature coefficient. As the temperature increases from 13–40°C. the highest level of opalescence reached becomes lower and the time required to reach this highest level becomes shorter. The rate of splitting of the molecule is apparently slow at 13°C. with 50 per cent urea as, after 69 hours in the ice box at this temperature the opalescence in the solutions after dialysis is almost as high as in solutions kept at room temperature for 12 hours, and higher than those kept at room temperature for 24 hours.

The rate of splitting must increase rapidly with temperature between 25 and 40°C., as at 40°C. the degree of opalescence begins to decrease long before it reaches a high value, and the maximum opalescence reached is only 3 foot-candles compared with 6 for solutions kept at 13°C.

These results seem to differ from those of Hopkins (7), where the amount of insoluble protein was still increasing after 3 hours at 37°C. But in his experiments he was using a 5 per cent albumin solution, and in such a solution the equilibrium between the two reactions (denaturation and splitting) would be different from that found in the much more dilute albumin solutions used in these experiments.

One may conclude, therefore, that when denaturation of egg albumin is measured by the amount of insoluble protein formed with 50 per cent urea, the

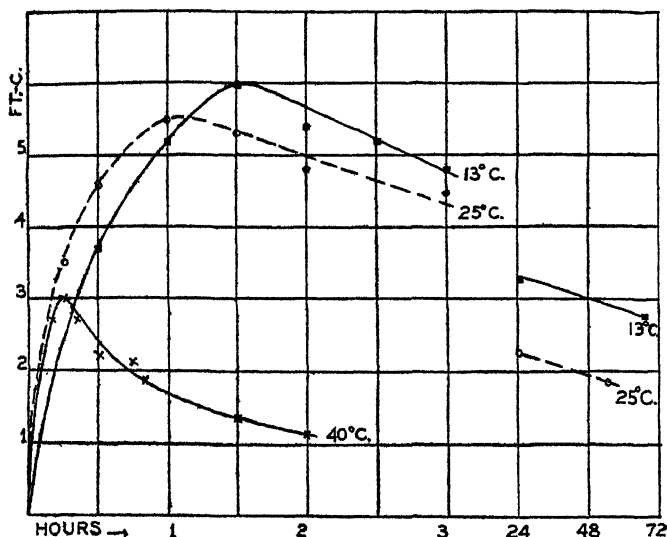


FIG. 1. Denaturation of egg albumin in 50 per cent urea at 13°, 25°, and 40°C. Ordinates = opalescence Tyndall beam in apparent foot-candles (a measure of the amount of insoluble protein present). Abscissae = time in hours.

results observed are due to two reactions, one increasing the amount of insoluble material (reaction 3), and the other decreasing it (reaction 4). As a result, the actual value of the temperature coefficient cannot be calculated, but it is obvious that both reactions have a positive temperature coefficient and that reaction 4 probably has a higher positive temperature coefficient than reaction 3, between 25 and 40°C.

*Denaturation with 40 Per Cent Urea.*—In Table II and Fig. 2 results are given for solutions containing 40 per cent urea, kept for varying periods of time at 13°, 25°, and 40°C. The figures are average values from a number of observations.

At this concentration the splitting of the molecule (reaction 4) is slower and reaction 3 proceeds fast enough so that at 40°C. the opalescence reaches its

maximum level, before any effect of splitting becomes apparent. The curves for the rate of development of opalescence at 13 and 25°C. are almost the same although the material at 25° reaches a maximum opalescence somewhat sooner. The fact that reaction 4 is progressing more rapidly at 25° is evident after the

TABLE II

Time	Amount of insoluble protein measured by Tyndall beam readings in apparent foot-candles		
	40°C.	25°C.	13°C.
15 min.	5.7		
30 "	6.4	2.75	
45 "	6.6		3.3
1 hr.	6.7	3.9	
1.5 "	6.2	4.6	4.3
2 "		5.4	5.1
3 "		6.0	
4 "		6.0	6.2
5 "		5.5	5.7
8 "		4.15	5.48
24 "		1.92	
48 "		1.15	4.4
72 "		0.72	3.3

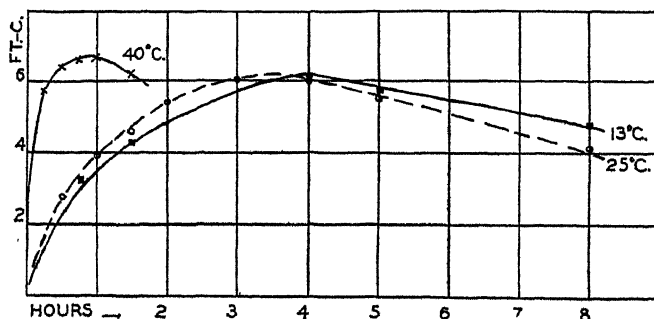


FIG. 2. Denaturation of egg albumin in 40 per cent urea at 13°, 25°, and 40°C. Ordinates = opalescence of Tyndall beam in apparent foot-candles (a measure of the amount of insoluble protein present). Abscissae = time in hours.

material has stood for a day or more. By that time the degree of opalescence in the material at room temperature has decreased very markedly while it is still high, though less than its maximum value, in the material at 13°C. The fact that the decrease in opalescence at 25°C. after several days is slightly greater in 40 per cent than in 50 per cent urea is probably not significant. The variations in room temperature over a period of several days would probably

be sufficient to account for this inconsistency, and actually fewer observations were made on material standing for several days, so that the 48 to 70 hour values are less accurate than those made up to 5 hours.

An additional fact of interest is this. If material which has stood for several days at either 13 or 25°C., until a large proportion of the molecules have been split, is then dialyzed and filtered until it is absolutely clear of suspended material, the filtrate will not precipitate on boiling. In other words, the changes produced by splitting are such that the change usually spoken of as denaturation (*i.e.* change in solubility) can no longer take place in the split molecules.

#### CONCLUSION

Evidence is brought forward to show that at concentrations of urea high enough to split the egg albumin molecule the solubility changes produced by urea are profoundly modified. The degree of precipitation after dialysis is the net result of two changes produced by the urea: the first, normally spoken of as denaturation, which makes the protein insoluble in dilute solution and the second, a splitting of the molecule which makes it soluble. These two reactions may proceed independently and simultaneously or the second reaction may follow the first, taking place in the denatured molecule only. In view of the decrease in the opalescence with time, the latter process is more probable.

Both of these reactions have positive temperature coefficients, but as the concentration of urea increases the second reaction is more affected by increase in temperature than the first, and consequently the resulting opalescence decreases rather than increases with temperature. This accounts for and explains reports of negative temperature coefficients of denaturation, when denaturation is measured by the amount of insoluble material found on dilution.

The occurrence of these two reactions, one leading to an increase and the other to a decrease in the amount of insoluble protein, should be taken into account when denaturation changes in egg albumin with urea are studied.

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# THE EFFECTS OF OXYGEN, CARBON DIOXIDE, AND PRESSURE ON GROWTH IN CHILOMONAS PARAMECIUM AND TETRAHYMENA GELEII FURGASON\*

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The effects of oxygen, carbon dioxide, and pressure on animal organisms have been widely studied, but most of the investigations in this field concern chiefly the effects of extreme, especially high, atmospheric pressures on blood changes, respiration, length of life, etc., in Vertebrates.

Very few detailed observations have been made on the relation between oxygen tension or carbon dioxide tension and growth in Protozoa. The few investigations that have been carried out are concerned primarily with the effect of these gases on respiration although some observations have been made on the relation of aerobic and anaerobic conditions and growth in unicellular forms.

Lwoff (1932) found that *Glaucoma piriformis* does not grow under anaerobic conditions, and Hall (1933) found that *Colpidium campylum* does not grow as well under anaerobic as under aerobic conditions. Jahn (1936) observed that *Chilomonas paramecium* grew best in unaerated culture flasks but that *Glaucoma piriformis* grew best in aerated flasks. The results of Phelps (1936) give support to those of Lwoff and Jahn on *Glaucoma piriformis*. Rottier (1936) maintained that inadequate amounts of oxygen limit the growth of *Polytoma uvella* and Reich (1936) claimed that oxygen concentration is very important in cultures of *Amoeba*.

Except for the work of Mast and Pace (1933) on the effects of carbon dioxide on growth in *Chilomonas paramecium* in inorganic solution, no observations have been made on the effect of various oxygen or carbon dioxide tensions on growth in unicellular forms. In the following pages, data concerned with the effects of different concentrations of these gases as well as the effects of total atmospheric pressure on unicellular organisms are presented.

## Material and Methods

Two very different species were used in these investigations; a colorless flagellate, *Chilomonas paramecium* and a small ciliate, *Tetrahymena geleii* Furgason.<sup>1</sup> The for-

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<sup>1</sup> *Tetrahymena geleii* Furgason, used in this work, was kindly furnished us by Professor George W. Kidder of Brown University. There is some controversy as to terminology, but in this report the genus will be referred to as *Tetrahymena*.

mer is plant-like and the latter animal-like in characteristics. Both species can be grown in sterile culture. The chilomonads were cultured in a solution of Na-acetate, 125 mg.;  $\text{NH}_4\text{Cl}$ , 46 mg.;  $(\text{NH}_4)_2\text{SO}_4$ , 10 mg.;  $\text{K}_2\text{HPO}_4$ , 20 mg.;  $\text{CaCl}_2$  1 mg.;  $\text{MgCl}_2$ , 1 mg.; thiamine hydrochloride, 0.001 mg.;  $\text{FeCl}_3$ , 0.001 mg.; and redistilled water to make 1 liter. The H ion concentration was adjusted to pH 6.8. This solution will be referred to as the acetate-ammonium or ac-am solution. Tetrahymenas were grown in a 2 per cent proteose-peptone solution, the H ion concentration of which was pH 6.8. Therefore no adjustment was necessary.

In these experiments, cultures were kept in large tightly sealed desiccators, which were connected by means of high pressure tubing to a vacuum pump and the cylinders containing the different gases. The connections were such that complete evacuation could be obtained and the desired gas or mixture of gases added without disconnecting the apparatus. In preparing cultures,  $2000 \pm 200$  chilomonads or tetrahymenas from a flourishing culture were added to each 70 ml. of fresh ac-am or proteose-peptone solution, respectively, in 125 ml. pyrex Erlenmeyer flasks. These were placed in desiccators. In order to test the effects of different gases of known concentration, the desiccators were evacuated by means of a Cenco pressovac pump, and the gas or mixture was added. The temperature was held at  $25 \pm 1^\circ \text{C}$ . for 4 days. The cultures were then removed, the density of the population calculated, and transfers were made to fresh solutions. The organisms were counted by diluting the culture with fresh ac-am solution (in thickly populated cultures the dilution was 1:100; otherwise, it varied according to density); then 0.1 ml. portions of the diluted culture fluid were added to Columbia dishes and the chilomonads counted under the dissecting microscope by drawing them into a micropipette and then discarding them. Six to ten counts were made on each culture. Very little variation was found.

## RESULTS

### *The Effect of $\text{O}_2$ Tension on Growth*

Since any variation in atmospheric pressure results in a proportionate variation in oxygen tension, results obtained from experiments designed to ascertain the effect of reduced pressures merit attention only if the  $\text{O}_2$  pressure is held constant. Consequently, the first experiments in these investigations were concerned with the optimum  $\text{O}_2$  concentration and the range of  $\text{O}_2$  concentrations in which *Chilomonas* and *Tetrahymena* continue to grow and reproduce. In each test, four 125 ml. Erlenmeyer flasks containing 70 ml. ac-am or proteose-peptone solution were used for each different  $\text{O}_2$  concentration; then  $2000 \pm 200$  chilomonads or tetrahymenas were added to each flask. The concentration of  $\text{O}_2$  varied from 0.5 mm. to pure  $\text{O}_2$  at atmospheric pressure ( $738 \pm 3$  mm.). The total pressure was kept constant for all the tests. This was done by evacuating the desiccator to approximately 3 mm. and adding the desired amounts of  $\text{O}_2$  and  $\text{CO}_2$  plus  $\text{N}_2$  to atmospheric pressure. The temperature was held at  $25 \pm 1^\circ \text{C}$ . After 4 days, the number of organisms in each culture was calculated and recorded. This test was repeated and the average

results for both tests are given in Table I, Experiment A. The experiment was then repeated and the averages for both tests are given in Experiment B.

The average results of both experiments are shown in order to emphasize the similarities in maximum numbers produced. Obviously, *Chilomonas* does not grow well in high concentrations of O<sub>2</sub>. At 600 mm. O<sub>2</sub> pressure there were comparatively few chilomonads found, and they died out on the first transfer. It can be concluded, then, that very little, if any, growth occurs in *Chilomonas* exposed to O<sub>2</sub> tensions above 500 mm. The optimum O<sub>2</sub> tension is approxi-

TABLE I

*The Effect of Oxygen in Various Concentrations on Growth in Chilomonas paramecium and Tetrahymena geleii*

To each 70 ml. of culture solution,  $2000 \pm 200$  organisms were added. Atmospheric pressure,  $738 \pm 3$  mm.; temperature,  $25 \pm 1^\circ$  C.

Concentration of O <sub>2</sub> at atmospheric pressure	No. of organisms per ml. after 4 days					
	<i>Chilomonas paramecium</i>			<i>Tetrahymena geleii</i>		
	Experiment A	Experiment B	Total average	Experiment A	Experiment B	Total average
<i>mm. Hg</i>						
0.5	102,400	98,000	100,200	13,000*	All dead	—
10	227,900	193,000	210,450	146,700	132,000	139,350
25	248,200	198,500	223,100	111,500	158,000	134,750
50	250,000	209,000	228,500	121,000	157,000	139,000
75	317,100	288,800	302,950	173,120	163,000	168,000
150	248,500	210,000	228,750	176,310	170,000	173,150
300	233,400	218,000	225,700	208,000	196,000	202,350
400	230,500	202,000	216,200	365,000	315,000	340,000
500	157,800	98,500	128,150	317,500	325,600	321,550
600	500*	600*	—	432,000	397,000	414,500
700	—	—	—	409,000	422,000	415,500
739	—	—	—	527,000	464,000	495,900
(Pure O <sub>2</sub> )						

\* Died after first transfer.

mately 75 mm. at which an average of 302,950 chilomonads per ml. was found for all tests. *Chilomonas* grew well even in O<sub>2</sub> tensions as low as 0.5 mm. This is in contrast to results obtained for *Tetrahymena*, which did not grow at these low O<sub>2</sub> concentrations. However, at 10 mm. O<sub>2</sub> pressure it grew very well and as the O<sub>2</sub> tension increased above this, maximum density of population increased until in pure O<sub>2</sub> 495,900 tetrahymenas were found in each milliliter of solution. These two species have very different respiratory systems. Notwithstanding, both will live under conditions in which they are exposed to a rather wide range of O<sub>2</sub> concentrations. *Chilomonas*, however, is killed at high O<sub>2</sub> tensions, while *Tetrahymena* grows best at high O<sub>2</sub> tensions. An interesting observation was



noted with regard to chilomonads grown in extremely low  $O_2$  concentrations (*i.e.*, when the gaseous environment consisted almost entirely of  $N_2$ ). Under these conditions they became very fragile and disintegrated unless great care was taken when preparing the organisms for counting.

#### *The Effect of Reduced Pressure on Growth*

In preliminary tests which were carried out to ascertain the lowest pressure under which these organisms would grow, it was found that *Chilomonas* grew best under slightly reduced pressures. However, in these tests the oxygen concentration was not held constant. The results of the experiments reported above show that *Chilomonas* grew best in  $O_2$  tensions lower than those found at atmospheric pressure. On the other hand, in preliminary tests, it was found that growth in *Tetrahymena* was retarded when pressure was reduced. The results of the experiments reported above for this organism show that the same is true when the oxygen tension is reduced. Could this increase in growth in *Chilomonas*, or retardation of growth in *Tetrahymena*, found when the pressure is reduced, be due entirely to lowered  $O_2$  tension? The following tests were carried out in an attempt to answer this question.

The same procedure was followed as in preceding tests except that the  $O_2$  tension was held at 50 mm. pressure in all tests. This particular pressure was selected for two reasons; first, both *Chilomonas* and *Tetrahymena* grow well at this concentration and, secondly, it is low enough to allow for considerable variation in pressure. The total pressures were varied from 50 mm. to atmospheric pressure, but the partial pressure of  $O_2$  was always 50 mm. Thus, at 50 mm. total pressure, there was pure  $O_2$  in the desiccator. The desiccators containing the cultures were kept at a fairly constant temperature,  $25 \pm 1^\circ C.$ , for 4 days when the number of organisms in the cultures was ascertained. The results are presented in Table II. They indicate that in *Chilomonas* there is no significant difference in growth over a wide range of pressures as long as the oxygen concentration is held constant. This answers the question that arose during the preliminary tests; *i.e.*, the question pertaining to the increase in growth observed when total pressure was reduced without regard to changes in  $O_2$  concentration. The increase in growth was apparently due to the low oxygen tension and not to the low pressure. In *Tetrahymena*, however, there is a significant increase in maximum numbers as the total pressure decreases. The optimum pressure seems to be at about 500 mm., since 280,000 organisms per ml. were produced as compared to 148,000 per ml. at atmospheric pressure. With further reduction in pressure, growth decreased until at 50 mm. an average of only 93,000 organisms per ml. was produced. The  $O_2$  tension used was far below the optimum for *Tetrahymena*, and therefore it can be concluded that the increase in growth was caused by some low pressure effect on the mechanism concerned with growth.

*The Effect of CO<sub>2</sub> Tension on Growth*

In most of the experiments previously reported the CO<sub>2</sub> was held constant at the concentration usually found at atmospheric pressures (approximately 0.03 per cent). Mast and Pace (1933) claimed that CO<sub>2</sub> is utilized by *Chilomonas* in the formation of starch, *etc.*, and maintained that when it is omitted from the surrounding air, the rate of growth is considerably reduced. Jahn (1936) confirmed the observations of Mast and Pace but found that in *Glaucoma piriformis* there was no difference in growth either in the presence or absence of CO<sub>2</sub>. In the following experiments, the effect of carbon dioxide on growth was ascertained in *Chilomonas* and *Tetrahymena*. The O<sub>2</sub> concentration was the same for all cultures (150 mm.) and the total pressure was always atmospheric (738

TABLE II

*The Effect of Reduced Pressure on Growth in Chilomonas paramecium and Tetrahymena geleii*

To each 70 ml. of culture solution, 2000  $\pm$  200 organisms were added. Each figure represents the average for 3 tests. Atmospheric pressure, 740 mm.; O<sub>2</sub> concentration, 50 mm.; temperature, 25  $\pm$  1° C.

Pressure  mm. Hg	No. of organisms per ml. after 4 days	
	<i>Chilomonas paramecium</i>	<i>Tetrahymena geleii</i>
50	195,000	93,300
100	190,000	—
200	180,800	118,700
300	185,100	122,000
400	185,300	214,000
500	192,000	280,000
600	202,100	204,000
740	191,000	148,000

mm.). The same procedure was followed as in previous experiments. The concentration of CO<sub>2</sub> ranged from that found in atmosphere (0.22 mm.) to 400 mm. CO<sub>2</sub>. The results are presented in Table III. This table shows that *Chilomonas* grew well in any of the concentrations used and that as the concentration of CO<sub>2</sub> was increased from zero, the growth increased to a maximum at 100 mm. CO<sub>2</sub> and then decreased. In *Tetrahymena*, growth was best in cultures exposed to CO<sub>2</sub>-free air. They did not live when exposed to CO<sub>2</sub> tensions much above 122 mm.; *i.e.*, they died out after the first or second transfer in the higher concentrations. Could these results have been influenced by the change in H ion concentration?

The H ion concentration at the optimum CO<sub>2</sub> tension (100 mm. CO<sub>2</sub>) is approximately pH 6.9. Mast and Pace (1938) found that the optimum H ion concentration for *Chilomonas* when grown in ac-am solution is pH 6.8.

It has been observed repeatedly that cultures of chilomonads become more alkaline as the population increases, but that when the CO<sub>2</sub> concentration is increased sufficiently, the solution becomes (or remains) acid. However, the

TABLE III

*The Effect of Carbon Dioxide in Various Concentrations at Atmospheric Pressure on Growth in Chilomonas paramecium and Tetrahymena geleii*

To each 70 ml. of culture solution, 2000  $\pm$  200 organisms were added. O<sub>2</sub> concentration, 150 mm.; pH, average H ion concentration of solutions after 4 days (pH varied by  $\pm$  0.1); temperature, 25  $\pm$  1° C.

Concentration of CO <sub>2</sub> at atmospheric pressure	No. of organisms per ml. after 4 days							
	<i>Chilomonas paramecium</i>				<i>Tetrahymena geleii</i>			
	Experiment A	Experiment B	pH	Total average	Experiment A	Experiment B	pH	Total average
mm. Hg								
0	92,000	87,000	7.5	89,000 $\pm$ 8,000	172,000	143,000	7.3	157,500 $\pm$ 17,000
0.2	225,000	200,000	7.4	212,500 $\pm$ 14,000	155,000	125,000	7.2	140,000 $\pm$ 19,000
2	216,400	214,500	7.2	215,000 $\pm$ 12,000	140,700	129,000	6.9	134,500 $\pm$ 11,000
12.2	454,000	358,000	7.3	406,000 $\pm$ 40,000	136,300	115,500	6.7	126,000 $\pm$ 4,000
50	698,000	570,000	7.1	634,000 $\pm$ 32,000	159,800	101,000	6.8	130,000 $\pm$ 7,500
100	654,000	678,200	6.9	668,600 $\pm$ 30,000	131,000	109,000	6.3	120,000 $\pm$ 6,000
122	574,700	519,000	6.9	546,000 $\pm$ 28,000	137,000	99,000	6.4	118,000 $\pm$ 4,800
244	325,000	219,500	6.2	272,000 $\pm$ 16,000	59,700 (Died out, 2nd transfer)	36,500	6.2	—
400	228,700	207,500	6.0	216,600 $\pm$ 9,000	34,900 (Died out, first transfer)	27,000	6.0	—

increased acidity produced by the addition of CO<sub>2</sub> had no significant effect on the results obtained in the experiment. This was proved by means of tests carried out to ascertain the extent to which the H ion concentration influenced the results. Several cultures of *Chilomonas* and *Tetrahymena* were set up at the same time and in the same way as those of the other tests. They were kept in air at ordinary atmospheric pressure but the H ion concentration was held at

the same pH value as those of other cultures which were exposed to CO<sub>2</sub>. The results are given in Table IV.

These results show that there is an increase in growth in *Chilomonas* as the H ion concentration increases (from pH 7.2–6.9) even in solutions to which HCl is added (to hold the pH on the acid side of neutrality). The increase, however, is hardly significant when compared to the increase in growth observed when the H ion concentration increases due to high CO<sub>2</sub> tensions. In other words, under atmospheric conditions, the average maximum number of chilomonads produced at pH 7.2 was 200,000 per ml. while at pH 6.9 (with HCl added) the maximum was 231,000. However, when the change in pH was caused by the addition of CO<sub>2</sub>, the maximum at pH 7.2 (2 mm. CO<sub>2</sub> pressure) was 215,000 per ml. and at 6.9 (100 ml. CO<sub>2</sub> pressure), 668,600 chilomonads per

TABLE IV

*The Relation between Carbon Dioxide and H Ion Concentration and Growth in Chilomonas paramecium and Tetrahymena geleii*

To each 70 ml. of culture solution, 2000  $\pm$  200 organisms were added. Temperature, 25  $\pm$  1° C.

H ion concentration	No. of organisms per ml. after 4 days			
	<i>Chilomonas paramecium</i>		<i>Tetrahymena geleii</i>	
	pH decreased by adding CO <sub>2</sub> (Table III)	pH decreased by adding HCl	pH decreased by adding CO <sub>2</sub> (Table III)	pH decreased by adding HCl
pH				
7.2	215,000	200,000	140,000	148,000
6.9	668,600	231,000	134,500	142,000
6.2	272,000	197,000	Died out	115,200

ml. Even at pH 6.2, many more chilomonads were produced in cultures exposed to CO<sub>2</sub> than in those which were not exposed to CO<sub>2</sub>. In *Tetrahymena*, when the H ion concentration was increased by adding HCl, the growth maximum decreased from an average of 148,000 per ml. at pH 7.2 to 115,200 at pH 6.2. Indications are that the organisms would continue to grow and reproduce indefinitely at this H ion concentration. If the CO<sub>2</sub> tension is raised enough (to 244 mm.) to increase the H ion concentration to pH 6.2, all the tetrahymenas died (Table III). Death was due to the high CO<sub>2</sub> concentration and only partly, if at all, to the comparatively high H ion concentration; in fact, *Tetrahymena* will live and grow in much higher H ion concentrations than this.

At frequent intervals, organisms were taken from the cultures at random and examined. Tests for starch and fat were made on chilomonads by means of diluted Lugol's solution (1 part in 20 parts water) and sudan III, respectively. Numerous specimens and their contents were traced by means of a camera lucida. Outlines of chilomonads so treated and drawn are given in Fig. 1.

Some of these representative specimens were grown in ac-am solution at ordinary atmospheric pressure while others were grown in ac-am solution with the optimum  $O_2$  and  $CO_2$  concentration for *Chilomonas*. The starch content was much greater in chilomonads grown under high  $CO_2$  pressures, and the starch grains were much larger than in the organisms grown in air at atmospheric pressure. The total starch produced by all the chilomonads (per unit volume) grown under optimum concentrations of  $CO_2$  and  $O_2$  was at least 4 to 5 times as great as that produced by all the chilomonads (per unit volume) grown in atmospheric air. Fat formation seemed to be at a minimum under these conditions. In some individuals grown in  $CO_2$  and  $O_2$  at optimum concentrations,

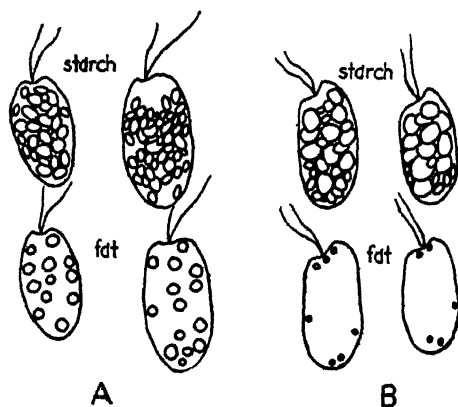


FIG. 1. Effect of high  $CO_2$  concentration on starch and fat content in *Chilomonas*. The chilomonads were selected at random and treated with Lugol's solution and sudan III. Outlines in upper sketches represent starch grains; those in lower sketches represent fat globules. A, camera lucida outlines of specimens cultured in ac-am solution in air at atmospheric pressure (737 mm.); B, chilomonads cultured in ac-am solution in  $O_2$  at 75 mm.,  $CO_2$  at 122 mm., and  $N_2$  at 540 mm. pressure (total pressure = 737 mm.).

no fat could be found. The low fat content is probably the result of the combined action of  $CO_2$  and  $O_2$ , for it has been observed that chilomonads grown in solutions exposed to high  $O_2$  tensions also contain very little fat.

In Fig. 2, several camera lucida sketches of *Tetrahymena* are shown. They were made from organisms which had been exposed to the same conditions as the chilomonads shown in Fig. 1. Numerous specimens were taken at random and stained with sudan III.

*Tetrahymenas* grown in pure oxygen show a decided increase in maximum numbers produced per unit volume (Table I). This is equivalent to approximately 3 times the maximum in atmospheric air. However, the organisms in pure  $O_2$ , as illustrated in Fig. 2, are much smaller than those in ordinary atmosphere. This is due to the greater rate of reproduction. Those grown

in high  $\text{CO}_2$  concentration were very small; sometimes less than one-fourth the volume of those in atmosphere. In other words, there was comparatively little growth in these organisms. In fact, it is quite likely that if the cultures had been continued under these high  $\text{CO}_2$  pressures, they soon would have died out. The tetrahymenas grown in air at atmospheric pressure contained a comparatively large number of fat globules which stain a deep orange-red with sudan III. These globules were located only at the anterior end of the organ-

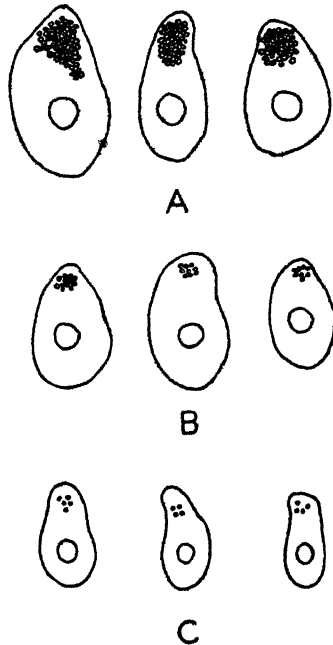


FIG. 2. Effect of  $\text{CO}_2$  and  $\text{O}_2$  on *Tetrahymena geleii*. Camera lucida sketches of organisms taken at random and stained with sudan III. Small circles represent fat globules. A, specimens from 2 per cent proteose-peptone solution in air at atmospheric pressure (737 mm.); B, specimens from solutions in pure  $\text{O}_2$  (737 mm.); C, specimens from solutions in  $\text{O}_2$  at 75 mm.,  $\text{CO}_2$  at 122 mm., and  $\text{N}_2$  at 540 mm. pressure (total pressure = 737 mm.)

isms. In pure  $\text{O}_2$  and in high  $\text{CO}_2$  plus  $\text{O}_2$ , the fat content decreased considerably. It is evident that the decrease in each condition was not caused by the same factor or factors. In pure  $\text{O}_2$ , the organisms divided very rapidly, oxidative metabolism was high, and fatty oxidation was stimulated. In high  $\text{CO}_2$  plus  $\text{O}_2$ , the tetrahymenas have evidently lost most of their power of synthesis, and thus very little protein, carbohydrate, or fat is formed.

*Chilomonas*, when grown in a favorable environment, contains a large quantity of stored food material in the form of starch granules and neutral fat. The presence of starch indicates that at least part of its metabolism is similar

to that found in plants. That its metabolic behavior is peculiar, compared to other animal cells, is now well known. Mast and Pace (1933) maintained that it produces starch, fat, and protein in a wholly inorganic medium (with one part of CO<sub>2</sub> added to 5 parts air at atmospheric pressure). Mast, Pace, and Mast (1936) obtained relatively low values for the respiratory quotient of *Chilomonas* which, they suggest, might be due to the reabsorption of the CO<sub>2</sub> which the chilomonads form during respiration and which may then be used in the resynthesis of starch. Hutchins (1941), however, disputes this claim and suggests that these low R.Q. values are due to incomplete absorption of the CO<sub>2</sub> produced in the Warburg flasks which were used for ascertaining respiration rate.

In the investigations reported here, the results indicate that *Chilomonas* differs considerably in its metabolism from the ciliate, *Tetrahymena*. The latter will not live in extremely high concentrations of CO<sub>2</sub> in which *Chilomonas* flourishes even when the carbon dioxide pressure reaches 400 mm. On the other hand, *Tetrahymena* grows well in high O<sub>2</sub> tensions and maximum growth is found in pure O<sub>2</sub> at atmospheric pressure (or possibly higher). *Chilomonas* does not grow well in O<sub>2</sub> concentrations above 500 mm. and its maximum is at 75 mm. O<sub>2</sub> (approximately one-half that of atmospheric air). Furthermore, it has been observed that in the higher CO<sub>2</sub> concentrations chilomonads not only show an increase in growth but also show an increase in the quantity of starch produced, and in the size of the individual starch granules which are extremely large compared to those produced in chilomonads grown in atmospheric air. Undoubtedly the increased synthesis of starch is enhanced by the presence of CO<sub>2</sub>. This is further evidence in support of the contention of Mast and Pace, that chilomonads utilize CO<sub>2</sub> in the synthesis of starch.

#### SUMMARY

1. The effects of O<sub>2</sub>, CO<sub>2</sub>, and pressure were studied in two very different species of protozoa, a flagellate, *Chilomonas paramecium*, grown in acetate-ammonium solution and a ciliate, *Tetrahymena geleii*, grown in 2 per cent proteose-peptone solution.

2. *Chilomonas* and *Tetrahymena* live and reproduce in solutions exposed to a wide range of O<sub>2</sub> concentrations, but *Chilomonas* is killed at high O<sub>2</sub> tensions in which *Tetrahymena* grows best. The optimum O<sub>2</sub> concentration for *Chilomonas* is about 75 mm. pressure but it lives and reproduces in O<sub>2</sub> tensions as low as 0.5 mm. while *Tetrahymena* fails to grow in concentrations below 10 mm. O<sub>2</sub> pressure.

3. With a constant O<sub>2</sub> tension of 50 mm. pressure, it was found that there is no significant variation in growth in *Chilomonas* between 50 mm. and 740 mm. total pressure. In *Tetrahymena*, however, under the same conditions, an optimum total pressure was found at about 500 mm. and growth is comparatively poor at 50 mm. total pressure.

4. *Tetrahymena* does not live very long in CO<sub>2</sub> tensions over 122 mm., although *Chilomonas* grows as well at 400 mm. CO<sub>2</sub> as in air at atmospheric pressure (0.2 mm. CO<sub>2</sub>). *Tetrahymena* grows best in an environment minus CO<sub>2</sub>, but the optimum for *Chilomonas* is 100 mm. CO<sub>2</sub> at which pressure an average of  $668,600 \pm 30,000$  organisms per ml. was produced (temperature,  $25 \pm 1^\circ$  C.).

5. Chilomonads grown in high CO<sub>2</sub> concentrations (e.g., 122 mm.) produce larger starch granules and more starch than those grown in ordinary air at atmospheric pressure.

6. In solutions exposed to 75 mm. O<sub>2</sub> tension (optimum) and 122 mm. CO<sub>2</sub> plus 540 mm. N<sub>2</sub> pressure, chilomonads contain very little, if any, fat. This phenomenon seems to be due to the action of CO<sub>2</sub> on the mechanisms concerned with fat production.

7. In *Tetrahymena* exposed to pure O<sub>2</sub>, there is very little fat compared to those grown in atmospheric air. This may be due to the greater oxidation of fat in the higher O<sub>2</sub> concentrations.

8. Further evidence is presented in support of the contention that *Chilomonas* utilizes CO<sub>2</sub> in the production of starch.

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# A PROTEOLYTIC ENZYME OF SERUM: CHARACTERIZATION, ACTIVATION, AND REACTION WITH INHIBITORS\*

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## INTRODUCTION

In previous reports (Christensen, 1944, 1945) evidence has been presented which indicates that the lysin factor of Milstone (1941) is an inactive proteolytic enzyme of serum which may be activated by streptococcal fibrinolysin, and that the activation is apparently catalytic in nature. It has also been shown that proteolysis by this activated enzyme is responsible for the phenomenon of streptococcal fibrinolysis.

Dastre (1893) originally drew attention to the proteolytic activity of serum, and a few years later Delezene and Pozerski (1903) reported that serum could be rendered proteolytic by treatment with chloroform. Since that time, many reports have appeared dealing with this chloroform-activated serum protease. In a series of recent papers, Tagnon and his coworkers (1942 *b, c*) have reviewed much of the literature. The serum protease may also be activated by other means, such as treatment with organic solvents or dialysis (Yamakawa, 1918), acid precipitation of the serum (Opie and Barker, 1908), and by treatment with certain organic compounds such as urea, benzoate, thiocyanate (Jühling and Wöhlisch, 1938), and cresols (Pope, 1938). The rôle of the serum protease in blood coagulation has been investigated by Ferguson (1939, 1940) and by Tagnon (1942 *a, b, c, d*).

Delezene and Pozerski (1903) observed that serum contains two opposing elements, the chloroform-activated protease, and an inhibitor of proteases, first described by Hildebrandt (cited by Opie, 1922). They were led to the conclusion that serum protease is normally inactive because of the action of the serum inhibitor, and that the function of the chloroform is to free the protease from it. The concept that serum protease becomes active because of the removal or inactivation of inhibitor has been held by several investigators. Yamakawa (1918) for example, suggested that the activation of serum protease upon treatment with organic solvents is due to removal of the inhibitor, a conclusion supported by the work of Teale and Bach (1919) who showed that inhibitor is removed from serum as denaturation of the serum proteins takes place. Schmitz (1937) interpreted the increase in activity which he noted when serum extracts were treated with alumina gel or dialyzed in dilute acetic acid to be due to the splitting of the inhibitor-enzyme complex, the inhibitor being adsorbable,

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acid-soluble, and dialyzable. Jühling and Wöhlisch (1938) concluded that the fibrinolysis which follows treatment of plasma with urea is due to removal of the inhibitor by urea. The literature on the serum inhibitor of proteases has been reviewed by Opie (1922) and by Grob (1943).

Schmitz (1938), employing the method used by Kunitz and Northrop (1936), isolated a small amount of inhibitor from serum which appeared to be similar to, if not identical with the crystalline trypsin inhibitor isolated by the latter workers from the pancreas.

There are certain similarities between fibrinolysin-activated lysin factor and chloroform-activated serum protease (Christensen, 1944, 1945; Kaplan, 1944). For example both are present in the euglobulin fraction of serum, both are present as inactive precursors which must be activated, and both are active against a variety of proteins at neutral pH.

Despite the similarities between chloroform-activated serum protease and fibrinolysin activated lysin factor, one striking difference may be noted. Activation of the serum protease by any of the recommended methods appears to be a relatively slow process, whereas in the presence of an excess of fibrinolysin, lysin factor is activated almost instantaneously. These observations have been confirmed recently by Kaplan (1944).

The data obtained in the present study indicate that the protease activated by chloroform and that activated by fibrinolysin are one and the same enzyme. It is also shown that the protease is not inactive because of combination with inhibitor, but because it is in an inactive, precursor state from which it can be activated by streptococcal fibrinolysin in a manner analogous to the activation of trypsinogen by enterokinase or the mold kinase of Kunitz (1938). Following activation, the active enzyme may be inhibited by serum inhibitor or crystalline trypsin inhibitor obtained from the pancreas. This inactivation, however, is not influenced by fibrinolysin. The characteristics of the serum enzyme as determined in the present study indicate that the protease is not identical with pancreatic trypsin.

### *Methods and Materials*

1. The preparation and partial purification of streptococcal fibrinolysin concentrates have been described in detail in a previous paper (Christensen, 1945). The concentrated fibrinolysin solutions used in the present study represent a 200- to 300-fold concentration of a culture supernatant.

2. The method of determining proteolytic activity has been described in detail in a previous paper (Christensen, 1945). The proteolytic unit used in the present paper is defined as the amount of protease which will cause a decrease of 1 per cent per minute in the specific viscosity of the reaction mixture of gelatin and enzyme. The rate of viscosity drop is linear for about the first 10 to 15 minutes and is directly proportional to the enzyme concentration over the ranges used in this study.

3. *Lysin Factor*.—Lysin factor preparations containing only small amounts of serum

inhibitor were prepared by dialyzing human serum<sup>1</sup> for 1 to 2 days against running tap water, followed by dilution with 2 volumes of distilled water. Upon acidification to pH 5.0–5.5 with acetic acid a flocculent precipitate quickly settled out. The precipitate was collected by centrifugation and dissolved in a volume of saline buffer at pH 7.4 equal to <sup>1</sup> the original serum volume.

4. *Activated Lysin Factor*.—One volume of concentrated lysin factor solution was mixed with 1 volume of 1:10 dilution of concentrated fibrinolysin and incubated at 37°C. for 10 minutes. Under these conditions all of the proteolytic activity of the lysin factor is liberated within the first few minutes. The activated lysin factor solutions are stable for several hours at room temperature and for much longer periods in the refrigerator. However, in the majority of the experiments reported in the present paper, lysin factor was activated immediately before use.

5. *Serum Protease*.—The method employed was essentially that described by Tagnon (1942 b), a modification of the process devised by Delezenne and Pozerski (1903). With lots of plasma of 50 to 100 ml., fairly consistent results were obtained. When the same method was used with 1 to 5 liter quantities of plasma, however, activation was not obtained consistently, and not more than 25 to 50 per cent of the preparations showed sufficient activity to be useable. The details of the preparative procedure were as follows: Plasma<sup>1</sup> was shaken with  $\frac{1}{10}$  volume of chloroform for 2 minutes. A fibrin clot formed within a few hours and dissolved again within 24 to 36 hours, although in some cases a longer time was required. The majority of active preparations were obtained by fractionation of the material 2 to 4 days after disappearance of the fibrin clot, although in one instance activity did not develop for more than a week. The chloroform-serum mixture was centrifuged for 1 to 2 hours and the supernatant decanted from the mixture of chloroform and denatured proteins. The supernatant was then treated exactly as in the method for the preparation of lysin factor, namely, acidification to pH 5.0–5.5 of the dialyzed and diluted material. The precipitate was dissolved in saline buffer in a volume equivalent to  $\frac{1}{10}$  the original plasma volume at pH 7.4.

It is evident from the foregoing that lysin factor and serum protease are present in the same fraction of serum, the euglobulin.

6. *Crystalline Trypsin*<sup>2</sup>.—The sample of crystalline trypsin contained about 50 per cent magnesium sulfate. A solution containing 1.0 mg. per ml. of the dry material was made up in 0.05N HCl. Solutions of the concentration desired for testing were made up immediately before use in saline buffer and adjusted to pH 7.4.

7. *Crystalline Trypsin Inhibitor*<sup>3</sup>.—The crystalline inhibitor preparation contained about 50 per cent magnesium sulfate. The dry material was dissolved in saline buffer,

<sup>1</sup> The majority of the human plasma used in the present study was made available by the Blood Donor Service of the New York Chapter of the American Red Cross. It represented for the most part samples of whole blood which could not be used by the Donor Service because of positive serology, a short collection, or because it was of a type not suitable for whole blood transfusion.

<sup>2</sup> The crystalline trypsin was supplied through the kindness of Dr. J. S. Fruton.

<sup>3</sup> The crystalline trypsin inhibitor was supplied through the kindness of Dr. J. H. Northrop and Dr. M. Kunitz.

pH 7.4, in a concentration of 1.0 mg. per ml. This stock solution is stable in the refrigerator for a week or more. Before use, dilutions were made to the required concentration in saline buffer.

8. *Serum Inhibitor Solution*.—Landsteiner (1900) and others (Hedin, 1904-05; Opie and Barker, 1907; Hussey and Northrop, 1923) have shown that the inhibitory activity of serum against proteases is associated with the albumin fraction. The serum inhibitor used in these experiments consisted of a twofold concentration of the albumin fraction of pooled human sera, partially purified by several reprecipitations followed by dialysis. The final solution was made up in saline buffer, pH 7.4.

9. *Quantitative Estimation of Serum Inhibitor*.—Grob (1943) has employed a method for the estimation of serum inhibitor based on its ability to inhibit the tryptic digestion of casein. In the present study serum inhibitor was measured by comparing the inhibitory action of the serum sample and a standard preparation of crystalline trypsin inhibitor on the tryptic digestion of gelatin. A standard inhibition curve was prepared by mixing 0.5 ml. of solutions containing varying amounts of a standard crystalline trypsin inhibitor solution with 0.5 ml. of a solution of a standard crystalline trypsin solution containing 0.01 mg. per ml. The mixtures were incubated for 15 minutes and the residual proteolytic activity determined by the method used for the determination of serum protease activity. When the logarithm of the residual proteolytic activity is plotted against the concentration of trypsin inhibitor, a straight line is obtained. In determining the inhibitory activity of an unknown solution, 0.5 ml. quantities of appropriate dilutions of the sample were mixed with 0.5 ml. of the standard crystalline trypsin solution and incubated for 15 minutes. The residual activity of the mixture is then determined, and by interpolation on the standard curve the inhibitory activity of the unknown can be expressed in terms of milligrams of the standard inhibitor preparation per milliliter of solution.

10. *Saline Buffer*.—This consisted of the borax-borate mixture described by Clark (1928, page 213), containing 0.9 per cent NaCl and adjusted to pH 7.4.

Determinations of pH were made with a glass electrode. The temperature in all experiments was controlled to  $\pm 0.1^\circ\text{C}$ . Merthiolate 1:10,000 or 1:20,000 was used as a preservative in all organic solutions.

## EXPERIMENTAL

### *Identity of Lysin Factor and Serum Protease*

Measurements of the pH of optimum activity, pH of maximum stability, and temperature-activity relationships were made in order to characterize the chloroform-activated serum protease and fibrinolysin-activated lysin factor. Under the experimental conditions, both of these proteolytic enzyme preparations were found to be identical in these respects.

1. *pH of Optimum Activity*.—Preparations of fibrinolysin-activated lysin factor and chloroform-activated serum protease in saline buffer were adjusted to various pH values by the addition of HCl or NaOH, brought to  $35^\circ\text{C}$ ., and mixed with gelatin at the same pH. The mixtures were immediately placed in Ostwald viscosimeters and the proteolytic activity determined by the

method described previously. The data obtained in these experiments are plotted in Fig. 1.

It is evident from Fig. 1 that the two systems are most active at the same pH, in a rather narrow range between pH 7.2 and 7.6, and both show the same rapid decrease in proteolytic activity as the pH is increased or decreased from this range.

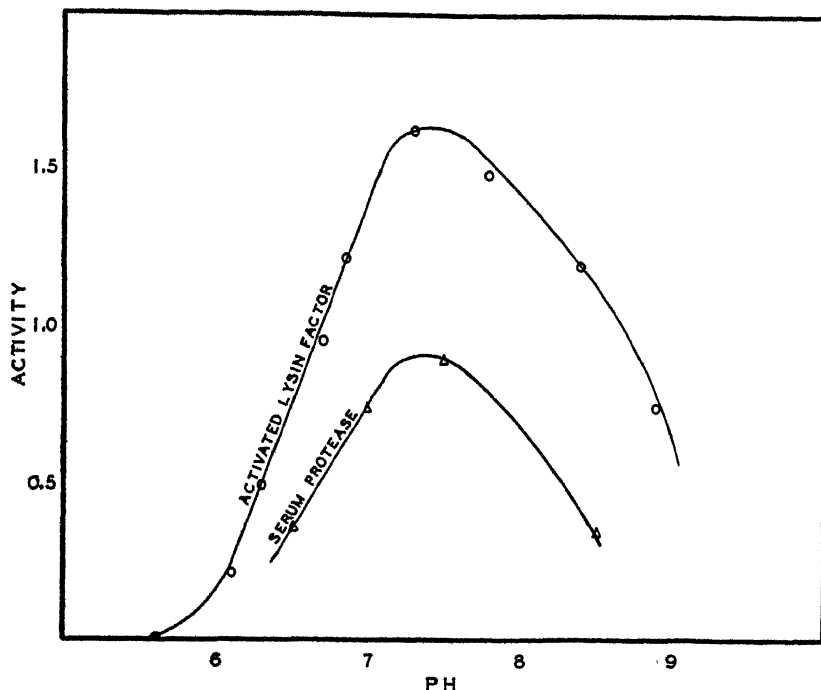


FIG. 1. pH of optimum activity of fibrinolysin-activated lysin factor and chloroform-activated serum protease. Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

2. *pH of Maximum Stability.*—In determining the pH of maximum stability of the two proteases, solutions of the fibrinolysin-activated lysin factor and chloroform-activated serum protease in saline buffer were adjusted to the desired pH values with HCl or NaOH and incubated at 45°C. for 30 minutes. At the end of this time they were removed, neutralized, adjusted to constant volume, brought to 35°C. as rapidly as possible, and tested for proteolytic activity against gelatin at pH 7.4. 45°C. was chosen as the inactivating temperature because the effects were more rapid than at lower temperatures, permit-

ting more observations to be made in a given time. The values obtained are shown in Fig. 2.

From the observations recorded in Fig. 2 it can be seen that at 45°C. both fibrinolysin-activated lysin factor and chloroform-activated serum protease have their maximum stability in a narrow range between pH 7.0 and 7.4, with rapid loss of activity above and below this pH range.

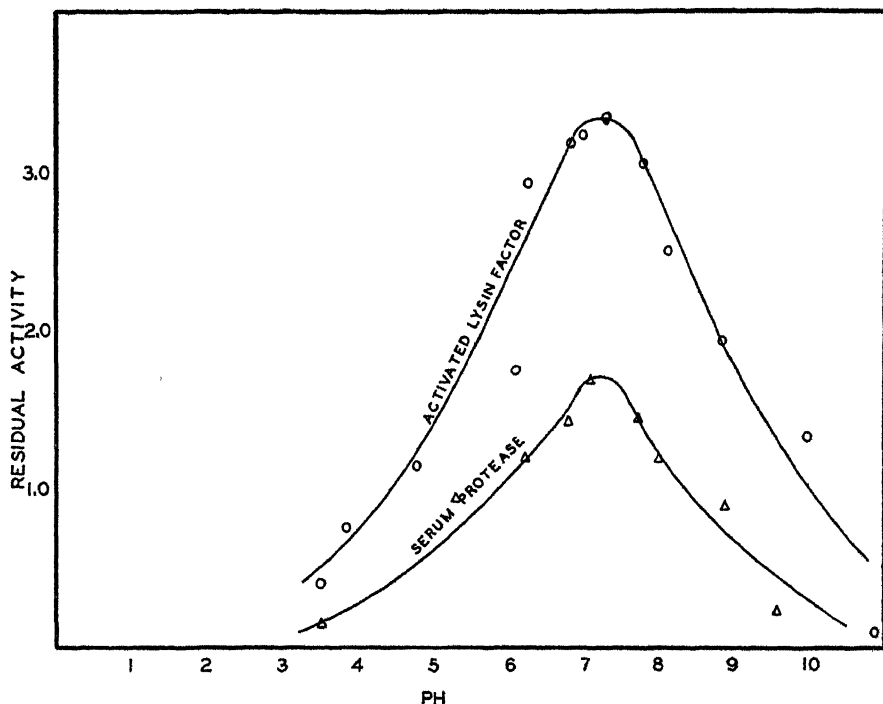


FIG. 2. pH of maximum stability of fibrinolysin-activated lysin factor and chloroform-activated serum protease at 45°C. Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

3. *Temperature-Activity Relationships.*—An Arrhenius plot of the temperature activity relationships of the two preparations was made. In determining the activity values for the Arrhenius plot, the proteolytic activity of the two preparations at the various temperatures was measured in the manner described, using gelatin at pH 7.4 as substrate. The temperature of each determination was controlled to within  $\pm 0.1^\circ\text{C}$ . In Fig. 3 the logarithms of the activities of the two proteases at the various temperatures have been plotted against the reciprocal of the absolute temperature.

The slopes of the curves for the values obtained with fibrinolysin-activated

lysin factor, as shown in Fig. 3, were calculated by the method of "least squares." Multiplication of arbitrary points on these calculated curves by a constant factor enables one to transpose the lysin factor curves to the experimental values obtained with chloroform-activated serum protease. When this is done, it can be seen that the lysin factor curves fit the serum protease values

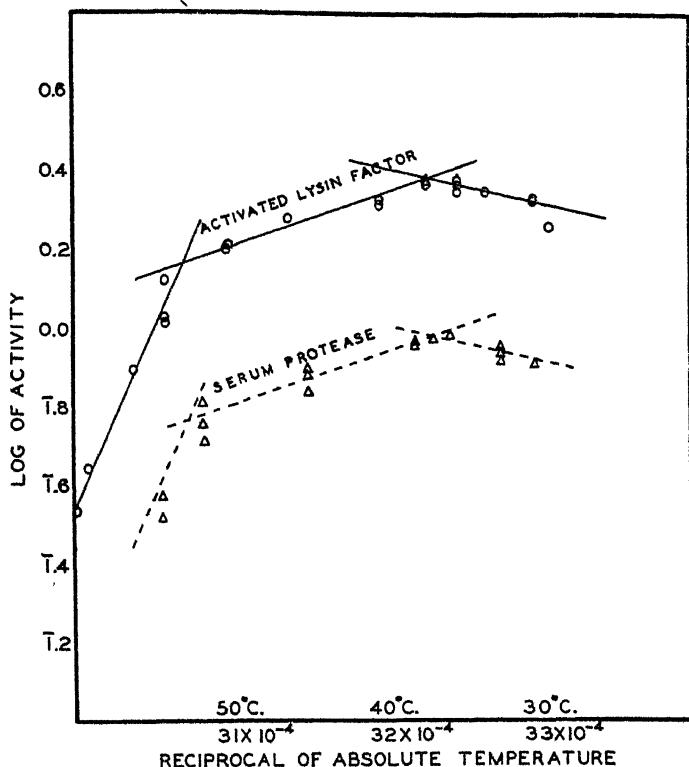


FIG. 3. Arrhenius plot of fibrinolysin-activated lysin factor and chloroform-activated serum protease. Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

quite well, indicating that the Arrhenius plots of the two preparations are identical. This calculation would appear to be valid because the slopes of an Arrhenius plot are independent of the units employed or the concentration of the enzyme, provided the units used are directly proportional to the enzyme concentration.

The Arrhenius plots presented in Fig. 3 indicate that the optimum temperature for the two preparations is about 37°C., as shown by the sharp change in direction of the plots at this point. Above 37°C. the activity decreases, pos-



sibly due to heat denaturation of the enzyme. A marked increase in the rate of inactivation is shown by both preparations as the temperature is raised above 53°C. The exact significance of this second break in the curve is not known; however, Sizer (1944) has recently reported a similar break in an Arrhenius plot of catalase.

It is possible to calculate the energy of activation and energy of inactivation of an enzyme from the slopes of an Arrhenius plot (Sizer, 1943). Calculation of these constants from the calculated slopes of the activated lysin factor plot indicates that the heat of activation is about 14,000 to 16,000 calories. The energy of inactivation in the region between 37° and 53°C. is about 50,000 calories, and in the region above 53°C. the energy of inactivation is about 400,000 calories. Because of inherent errors in the methods used, these figures are only approximations of the true values. For example, the viscosity of gelatin is not a regular function of temperature, nor does the viscosity of gelatin remain constant at higher temperatures in the absence of enzyme. Further, when gelatin is used as substrate, viscosity measurements cannot be made with any accuracy below a temperature of 30–32°C. Despite these sources of error, the energies of activation and inactivation are of the order of magnitude of many enzymes (Sizer, 1943).

The close similarity between fibrinolysin-activated lysin factor and chloroform-activated serum protease in respect to the effects of pH and temperature on their activity leaves little doubt that the two are very similar if not identical. In subsequent portions of the present paper, therefore, the term "serum protease" will be used to designate the proteolytic activity of serum, and unless otherwise specified will indicate fibrinolysin-activated material.

#### *Activation of Serum Protease by Fibrinolysin*

A study of the fibrinolysin activation of serum protease is accompanied by certain difficulties. Of these, the most important is that caused by the presence of variable amounts of serum inhibitor in the serum protease preparations, resulting in an apparent alteration in the course of the activation reaction. Furthermore, since no method has been found of destroying or inactivating fibrinolysin without at the same time destroying serum protease, some further activation of serum protease occurs during the period of testing for liberated serum protease activity. For these reasons the values obtained in the present study are only approximations of the true values.

These difficulties were minimized as much as possible by studying the activation of a sample of serum protease which contained only a small amount of serum inhibitor. The effect of continued activation during testing was minimized by activating with dilute solutions of fibrinolysin, so that the further 14-fold dilution of the fibrinolysin which occurs during the determination of proteolytic activity of the mixture would lower the concentration of fibrinolysin below an effective level.

1. *Activation of Serum Protease by Fibrinolysin in the Presence of Serum Inhibitor.*—Kunitz (1938) has shown that the formation of trypsin from trypsinogen by the mold kinase follows the course of a catalytic unimolecular, or first order reaction, which may be expressed by the equation

$$\log \frac{Ae}{Ae - A} = (KM)t$$

where  $Ae$  is the trypsin activity after complete activation,  $A$  is the concentration of active trypsin in the activation mixture at any time  $t$ , and  $(KM)$  is the slope of the straight line obtained when the values of

$$\log \frac{Ae}{Ae - A}$$

are plotted against the values for  $t$ .

In testing the activation of serum protease, aliquots of inactive serum protease preparations (lysin factor) were mixed with equal volumes of dilutions of a fibrinolysin concentrate and incubated at 25°C. in a water bath. At intervals, 1 ml. of the mixture was removed and tested for proteolytic activity. Two serum protease preparations were used in this study. Both had the same amount of proteolytic activity when treated with an excess of fibrinolysin; 3.0 proteolytic units per ml. of activation mixture. Preparation 514 contained serum inhibitor equivalent to 0.001 mg. per ml. of the standard trypsin inhibitor preparation, as determined by the procedure described in the section on methods. The second preparation, No. 598, contained inhibitor equivalent to 0.0047 mg. per ml. The data obtained in these experiments were analyzed by means of the equation used by Kunitz (1938) and the results plotted in Fig. 4.

It is evident from Fig. 4 that the activation of serum protease preparation 514, containing the least amount of inhibitor, is a linear function of the time of incubation, indicating a first order reaction (Kunitz, 1938). With the highest dilutions of fibrinolysin, 1:400 and 1:500, the curves deviate somewhat from a linear relationship, probably due to the action of serum inhibitor on the small amount of free enzyme which would be present at any time in the mixture. This conclusion is borne out by the results obtained with the second serum protease preparation, No. 598, which contains about 5 times as much inhibitor as preparation 514. In this case a linear relationship is not obtained, even with the higher concentrations of fibrinolysin.

It is apparent from the above data, therefore, that the kinetics of fibrinolysin activation of serum protease is not that of a first order reaction in the presence of serum inhibitor. However, as the concentration of inhibitor decreases, the kinetics approaches that of a first order reaction, suggesting a catalytic type of

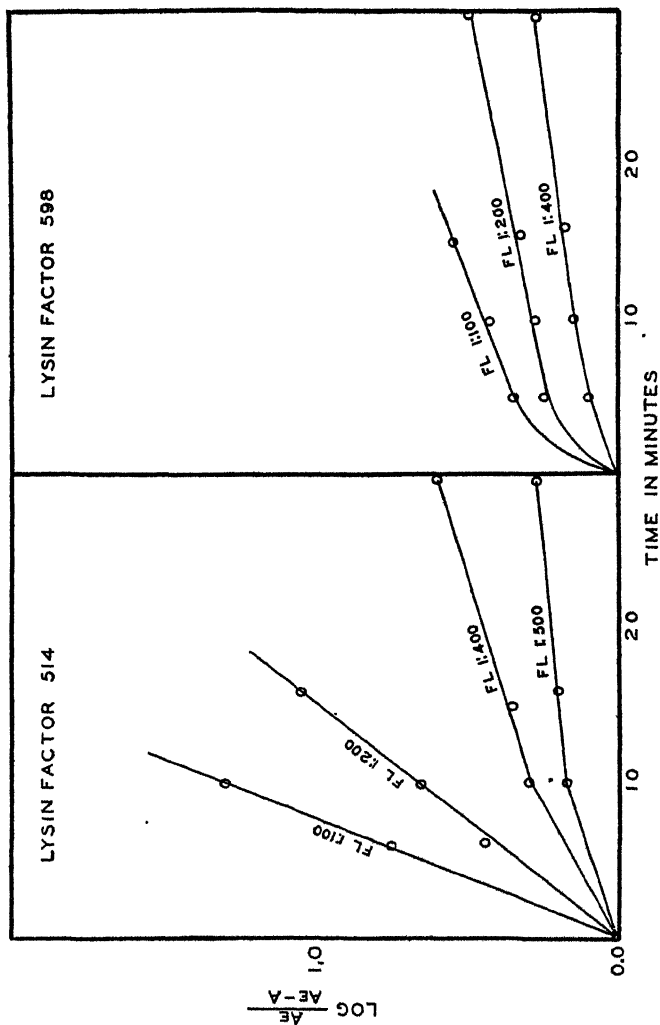


FIG. 4. Rate of activation of serum protease by streptococcal fibrinolysin. The left-hand chart represents the activation of preparation 514, which contained 0.001 mg. inhibitor per ml. The right-hand chart represents the activation of preparation 598, which contained 0.0047 mg. of inhibitor per ml. Both preparations possessed the same amount of proteolytic activity when activated by an excess of fibrinolysin.

activation of the serum protease by fibrinolysin, analogous to the kinase activation of trypsinogen.

2. *Effect of Fibrinolysin Concentration on Rate of Activation.*—In Fig. 4 the slopes of the lines are proportional to the rate of activation (Kunitz, 1938). In Table I are presented the slopes of the activation curves of serum protease preparation 514 when activated by varying concentrations of fibrinolysin, calculated from the data presented in Fig. 4.

From the calculations shown in Table I it can be seen that the slopes of the activation curves of the serum protease preparation are directly proportional to the concentration of fibrinolysin, except in the case of the more dilute preparations of fibrinolysin. The lack of proportionality in these cases is probably due to the presence of serum inhibitor in the sample, as has been discussed above. This is borne out by the fact that in the presence of higher concentra-

TABLE I  
*Effect of Fibrinolysin Concentration on the Rate of Activation of Serum Protease*

Fibrinolysin concentration	Slope of activation curve*
1:100	0.065
1:200	0.0325
1:400	0.015
1:500	0.006

\* The slope of the curve is a measure of the rate of activation.

tions of serum inhibitor than are present in preparation 514 (e.g. preparation 598), the rates of activation are not proportional to the fibrinolysin concentration, even when higher concentrations of fibrinolysin are used. On the other hand, with low concentrations of serum inhibitor the activation rates are more nearly proportional to the concentration of fibrinolysin. It appears highly probable, therefore, that in the absence of serum inhibitor, the rate of activation of serum protease by fibrinolysin would be directly proportional to the concentration of fibrinolysin, suggesting an enzymatic type of activation.

3. *Temperature Coefficient of Activation.*—The temperature coefficient of the activation reaction was determined by mixing aliquots of serum protease preparation 514 with equal volumes of a 1:200 dilution of fibrinolysin and incubating the mixtures at 25°C. and 35°C. At intervals, 1 ml. of the mixture was removed and tested for proteolytic activity. The results obtained are plotted in Fig. 5 in the same manner as in the previous experiment, using the equation of Kunitz (1938).

Calculation of the slopes of the curves in Fig. 5 reveals that an increase in temperature of 10°C. between 25° and 35°C. results in an increase of 1.76-fold in the rate of activation, indicating that the temperature coefficient of the reaction between these temperatures is about 1.8.

The data obtained in the above experiments are compatible with the hypothesis that the activation of serum protease by fibrinolysin is an enzyme-catalyzed reaction. The kinetics of activation is those of a first order reaction with the rate of activation directly proportional to the fibrinolysin concentration and the temperature coefficient of the reaction about 1.8 between 25° and 35°C. In the presence of serum inhibitor, however, the reaction appears to be complicated by combination of active enzyme with inhibitor, which causes the kinetics to differ from that of a first order reaction.

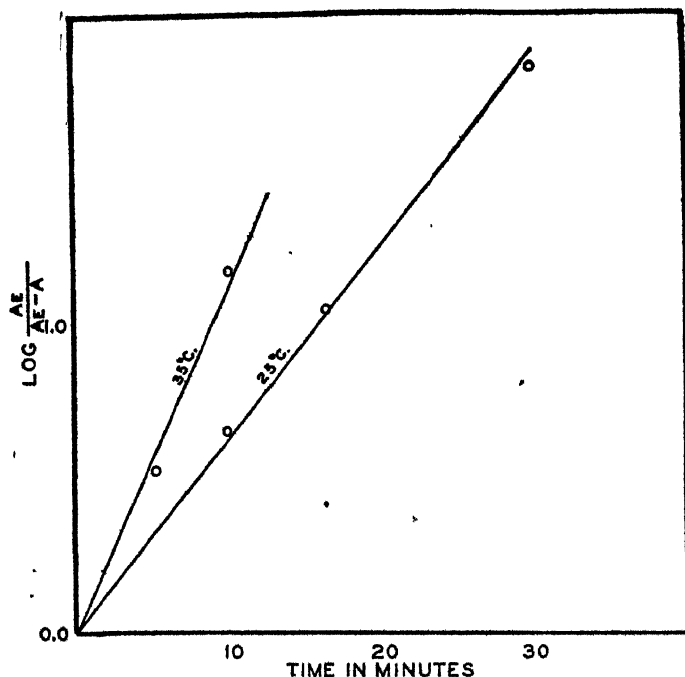


FIG. 5. The effect of temperature on the rate of activation of serum protease by streptococcal fibrinolysin.

#### *Interaction of Serum Protease and Inhibitors*

Following the demonstration that the activation of serum protease is an enzymatic type of reaction, it became of interest to determine the mechanism of activation. Mention has been made in the introduction to this paper of the hypothesis that serum protease is normally inactive in serum because of the presence of serum inhibitor.

1. *Nature of Serum Protease Inhibition by Crystalline Trypsin Inhibitor and Serum Inhibitor.*—Northrop and his coworkers have shown that both pancreatic trypsin inhibitor and serum inhibitor combine with trypsin, and that the kinetics of the reaction indicate the formation of an equimolar, dis-

sociable, inhibitor-enzyme compound (Northrop, 1939; Kunitz and Northrop, 1936; Hussey and Northrop, 1923). In order to determine whether or not the inhibition of serum protease is of similar nature, the inhibition of activated serum protease by crystalline pancreatic trypsin inhibitor and by serum inhibitor was compared with the effect of these agents on crystalline trypsin.

Fibrinolysin-activated serum protease was mixed with equal volumes of crystalline trypsin inhibitor solution of varying concentrations. The mixtures were incubated at 35°C. for 10 minutes to allow combination, and 1 ml. of the mixture was then tested for residual proteolytic activity against gelatin. A trypsin solution containing 0.01 mg. per ml. of the crystalline trypsin preparation was tested with inhibitor solutions in the same way. This concentration of trypsin was chosen because it possessed about the same proteolytic activity under the conditions of the present experiment as the activated serum protease preparations. The results of these experiments are plotted in Fig. 6. The curve drawn through the values obtained for the inhibition of trypsin was recalculated from the data of Northrop (1939, p. 11) on the inhibition of trypsin by trypsin inhibitor.

It is evident from the observations shown in Fig. 6 that the reaction between serum protease and crystalline trypsin inhibitor differs markedly from the reaction of trypsin with the inhibitor. Not only is the inhibitor much less active against serum protease than against trypsin, but the shapes of the two inhibition curves are so different as to suggest a marked qualitative difference in the inhibition of the two enzymes by crystalline trypsin inhibitor.

The experiment described above was repeated except for the substitution of serum inhibitor for the crystalline trypsin inhibitor, although available evidence suggests they are the same substance (Schmitz, 1938; Grob, 1943). The results are plotted in Fig. 7, with the curve drawn through the values obtained with trypsin recalculated from the data of Hussey and Northrop (1923) on the inhibition of trypsin by serum.

The data recorded in Fig. 7 indicate the same qualitative and quantitative differences in the inhibition of serum protease and trypsin by serum inhibitor as were found in the experiments employing the crystalline trypsin inhibitor from the pancreas.

Experiments have been carried out with chloroform-activated serum protease using the two inhibitors. The chloroform-activated protease behaved in the same manner as the fibrinolysin-activated material.

It is apparent, therefore, that although both crystalline trypsin inhibitor from the pancreas and serum inhibitor affect the activity of serum protease, the reaction is not the same as when these inhibitors act on crystalline trypsin.

*2. Effect of Fibrinolysin on Serum Protease Inhibition.*—It has been assumed by several investigators that serum protease is normally inactive because of the presence of serum inhibitor (Delezené and Pozerski, 1903; Yamakawa, 1918; Schmitz, 1937; Jühling and Wöhlisch, 1938). If this were correct it should be

possible to demonstrate an action of fibrinolysin on either the isolated inhibitor or on the inhibitor-enzyme complex.

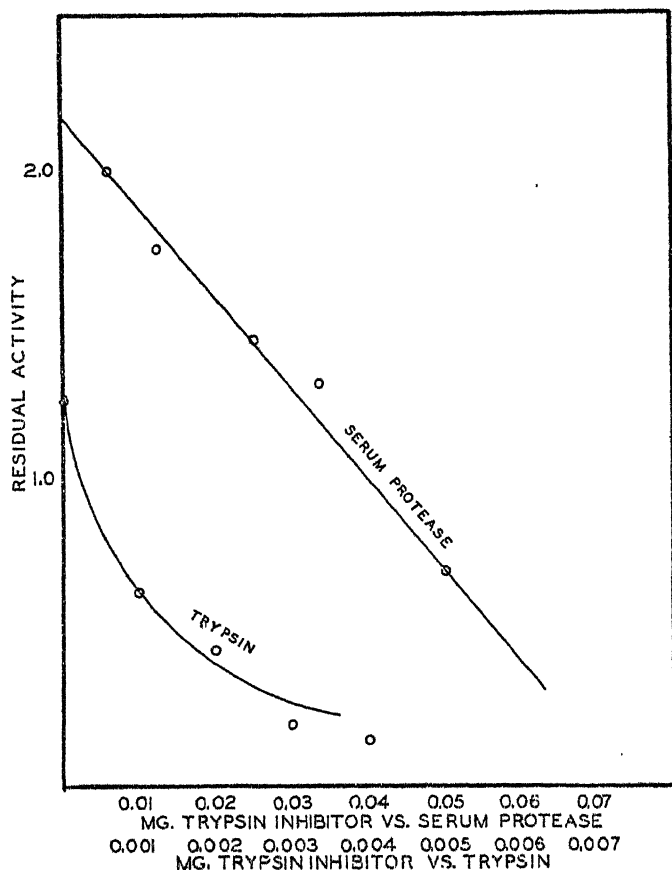


FIG. 6. Inhibition of serum protease and crystalline trypsin by crystalline trypsin inhibitor. The concentrations of inhibitor used with trypsin are only  $\frac{1}{10}$  those used to inhibit serum protease. The curve drawn through the values for crystalline trypsin was recalculated from data of Northrop (1939) and superimposed on the experimental points. Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

A sample of lysin factor was activated by adding a small amount of fibrinolysin solution and allowing the mixture to stand at room temperature for several hours. When activation was complete, the activated protease was salted out by the addition of  $\frac{1}{2}$  volume of saturated ammonium sulfate. The precipitate was collected by centrifugation, washed several times with  $\frac{1}{3}$  saturated ammonium sulfate solution, and reprecipitated. It was then dialyzed sulfate-free and made up to the original volume

in saline buffer. This procedure was employed in order to reduce the residual fibrinolysin concentration of the solution as much as possible.

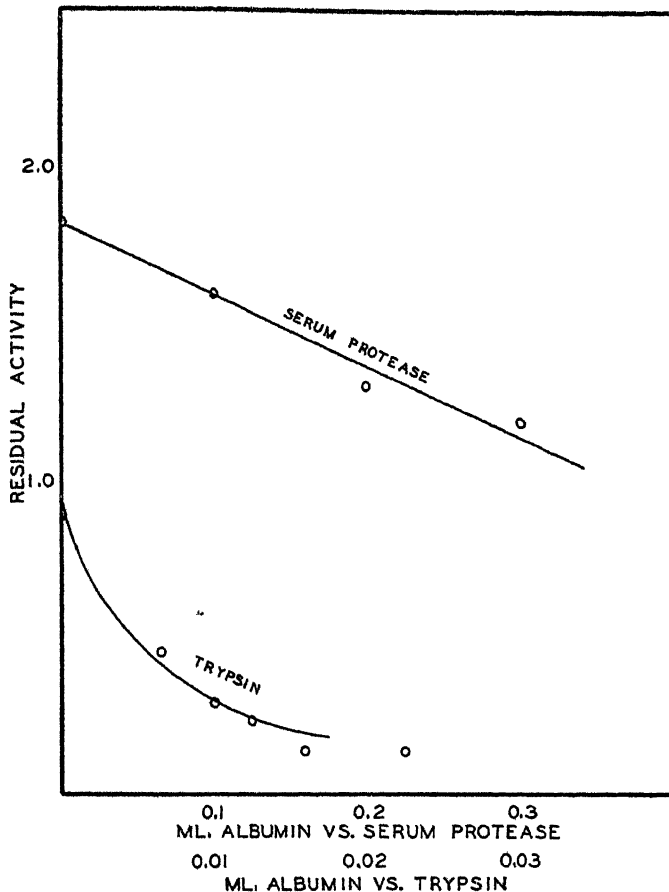


FIG. 7. Inhibition of serum protease and crystalline trypsin by serum inhibitor. The amounts of serum inhibitor (albumin solution) used to inhibit crystalline trypsin are  $\frac{1}{10}$  those used to inhibit serum protease. The curve drawn through the values obtained for crystalline trypsin was recalculated from data of Hussey and Northrop (1923) on the inhibition of trypsin by serum, and was superimposed on the experimental points. Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

In testing the effect of fibrinolysin on the inhibition of the activated protease, a series of tubes was set up, each containing 0.5 ml. of activated protease, 0.5 ml. of a solution of crystalline trypsin inhibitor, and 0.1 ml. of dilutions of a fibrinolysin concentrate. The mixtures were incubated at 35°C. for 10 minutes and tested for residual proteolytic activity. Control tubes contained respectively serum protease alone, serum protease with inhibitor, and serum protease with fibrinolysin. In order



to avoid an excess of inhibitor, the amount of crystalline inhibitor used was insufficient to inhibit the protease completely. The results of this experiment are shown in Table II.

As shown in Table II, under the experimental conditions employed fibrinolysin did not increase the activity of the partially inhibited protease. The lack of an activating effect of fibrinolysin does not appear to be due to insufficient fibrinolysin since it was observed repeatedly that the same fibrinolysin preparation was capable of activating completely within 10 minutes all lysin factor preparations with which it was tested.

TABLE II  
*Effect of Fibrinolysin on the Inhibition of Serum Protease by Crystalline Pancreatic Trypsin Inhibitor*

Inhibitor concentration	Residual activity after incubation in proteolytic units			
	Concentration of fibrinolysin			
	0	1:10	1:100	1:1000
<i>mg. per ml.</i>				
0	1.66	1.20	—	1.54
0.02	1.38	0.72	0.98	1.20

TABLE III  
*Effect of Fibrinolysin on Inhibition of Serum Protease by Serum Inhibitor*

Inhibitor concentration	Residual activity after incubation in proteolytic units			
	Concentration of fibrinolysin			
	0	1:10	1:100	1:1000
<i>ml. of albumin</i>				
0	1.66	1.20	1.42	1.60
0.2	1.3	1.02	1.37	1.45
0.5	0.9	0.72	0.77	0.78

It can also be seen from the data shown in Table II that the fibrinolysin solutions, rather than increasing the activity of the partially inhibited protease, actually caused a decrease in the residual activity, suggesting the presence of an inhibitor of proteases in the fibrinolysin solution itself. Such an inhibitor of proteases could be demonstrated in fibrinolysin preparations by adding them to trypsin solutions.

The experiment described above was repeated except for the substitution of serum inhibitor for crystalline trypsin inhibitor. The results of this experiment are shown in Table III.

As in the previous experiment, the data presented in Table III indicate that fibrinolysin does not increase the activity of the partially inhibited protease.

Likewise, it appears that the fibrinolysin solution itself caused some inhibition of the protease.

A number of variations of these experiments has been carried out. Incubation of fibrinolysin and inhibitor before addition of the protease was found to be without effect on the degree of inhibition. Furthermore, incubation of the protease and inhibitor, or protease and fibrinolysin before the addition of the third reagent was likewise without effect on the degree of inhibition. Chloroform-activated protease behaved in the same way as the fibrinolysin-activated material, that is, fibrinolysin did not decrease the amount of inhibition caused by either inhibitor.

It has also been found that the inhibition of crystalline trypsin by either serum or pancreatic inhibitor is not influenced by the presence of fibrinolysin in the reaction mixture, although again the fibrinolysin solutions inhibit the proteolytic activity of the trypsin to some extent.

It appears, therefore, that streptococcal fibrinolysin is entirely without effect on the inhibition of serum protease by serum or pancreatic inhibitors. Furthermore, since fibrinolysin is capable of activating serum protease from its normal inactive state in serum, it follows that this inactive state of serum protease is not due to combination of the protease with serum inhibitor, but to some other mechanism.

#### *Substrate Specificity of Serum Protease and Trypsin*

The reactions of serum protease and trypsin with protease inhibitors, together with the characteristics of serum protease as described in a previous section of the present paper, suggest that serum protease and trypsin are dissimilar enzymes.

Northrop (1939, p. 76) has shown that when an enzyme is allowed to act on a protein substrate until hydrolysis has virtually ceased, addition of a second quantity of the same enzyme does not result in further hydrolysis. On the other hand, if a different enzyme is added, further hydrolysis of the substrate takes place. This phenomenon is probably an expression of the different peptide bond specificities of the two enzymes, since, as Bergmann and his coworkers (1941, 1942) have shown, each proteolytic enzyme is characterized by the ability to hydrolyze certain specific peptide linkages. In Northrop's experiment, therefore, once all of the bonds susceptible to one enzyme are split, no further action can be produced by this enzyme although further hydrolysis will occur on the addition of an enzyme having different specificities.

In order to determine whether or not the serum enzyme and trypsin attack the same linkages of the substrate molecule, 20 ml. aliquots of a 5 per cent casein solution were mixed with 5 ml. portions of a solution of either serum protease or trypsin. At intervals, 1 ml. portions of the mixtures were removed, precipitated with trichloroacetic acid, and the acid-soluble tyrosine in the filtrate determined by a method outlined in a previous paper (Christensen, 1945). The following day, when the rate of acid-

soluble tyrosine liberation had dropped to nearly zero, the reaction mixtures were divided into three portions. In one portion the rate of acid-soluble tyrosine liberation was followed without the addition of other reagents; to the second portion was added a second quantity of the enzyme first used; to the third portion of the reaction mixture containing trypsin, serum protease was added, and to the reaction mixture containing serum protease, trypsin was added. The results obtained in this experiment are plotted in Fig. 8, where the milligrams of acid-soluble tyrosine liberated per milliliter of reaction mixture are plotted against the incubation time.

It is evident from the data presented in Fig. 8 that serum protease rather quickly liberates from casein the majority of the acid-soluble tyrosine which it is capable of liberating and addition of fresh enzyme does not result in any further increase. However, if trypsin is added to the serum protease digest, a prompt and marked rise in acid-soluble tyrosine liberation occurs, indicating that linkages are still available for the action of trypsin after all those susceptible to serum protease have been split. On the other hand, if casein is first digested by trypsin, addition of serum protease to the digest mixture does not result in further hydrolysis of the casein, although the increase in acid-soluble tyrosine on addition of more trypsin indicates that hydrolysis by trypsin is not yet complete. This might be taken as an indication that trypsin is able to hydrolyze not only the linkages split by serum protease, but others as well. In any case, it is evident that trypsin is able to continue hydrolysis of casein after hydrolysis by serum protease has gone to completion.

#### DISCUSSION AND SUMMARY

The observations recorded in the present paper indicate that the lysin factor described by Milstone (1941) and the chloroform-activated serum protease are one and the same proteolytic enzyme, differing only in their mode of activation. The serum protease is most stable at a pH near 7.2, and its pH of optimum activity is also in this region. The pH of optimum activity may be not so much a reflection of the pH at which the individual molecules of the enzyme are most active, but instead be related to the marked instability of the enzyme above and below this pH range. The Arrhenius plot of the temperature-activity characteristics of the enzyme indicates that the optimum temperature for activity is about 37°C. Above this temperature activity falls off rapidly, probably due to heat inactivation. A second sharp break in the Arrhenius plot occurs at a temperature of about 53°C., above which point the activity of the enzyme decreases with great rapidity. This type of two-phase inactivation curve for an enzyme has recently been discussed by Sizer (1944) in relation to a similar phenomenon which occurs with catalase. The energies of activation and inactivation of the enzyme, as calculated from the slopes of the Arrhenius plot, are only approximations because of certain errors inherent in the methods used. However, the values obtained, 16,000 calories and 50,000 calories respectively, are of the order of magnitude characteristic of most enzymes. The energy of inactivation above 53°C., about 400,000 calories,

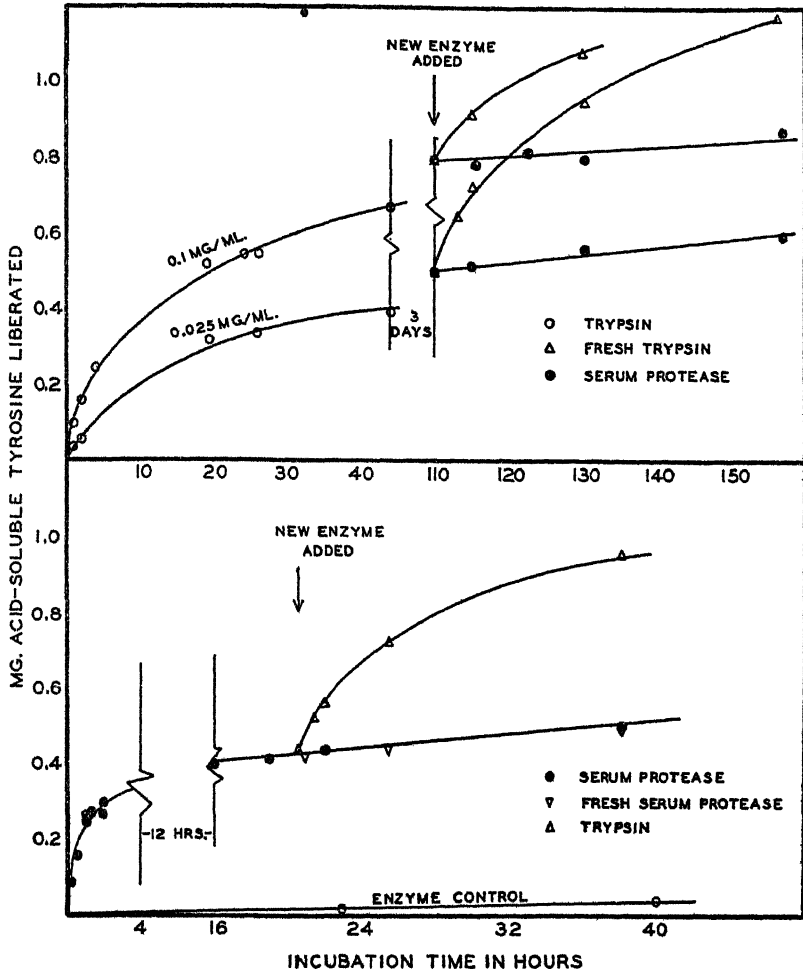


FIG. 8. Combined action of serum protease and trypsin on casein. The upper portion of the figure represents the hydrolysis of casein by trypsin, followed by the addition of fresh trypsin solution (containing 0.5 mg. of the dry material per ml.) and serum protease solution (containing about 2.8 proteolytic units per ml.). The lower portion of the figure represents the hydrolysis of casein by serum protease solution (2.8 proteolytic units per ml.), followed by the addition of fresh serum protease solution and by trypsin solution (0.5 mg. per ml.). Activity is expressed in terms of proteolytic units, determined as described in the section on methods.

is higher than the values recorded bySizer (1943) for most other enzymes. The actual value is subject to some error, but the order of magnitude for the inactivation energy of serum protease above 53°C. agrees with the higher value found for catalase bySizer (1944), namely 255,000 calories.

It seems apparent that serum protease is not trypsin nor is it similar to trypsin in its properties, as has been implied previously (Schmitz, 1936, 1937; Iyengar, 1942; Ferguson, 1939, 1940 *a*, *b*; Tagnon, 1942 *a*, *b*). Trypsin, in contrast to serum protease, does not exhibit as sharp a zone of maximal activity in the neighborhood of pH 7.4, with the marked drop in activity above and below this point that is shown by serum protease. Crystalline trypsin is most stable at a pH of about 3, but crude preparations are stable over a range of about pH 6-8. Since the serum protease preparation is quite crude, it is not possible to state that the two enzymes differ in this respect.

Other evidence of the non-identity of the two enzymes, trypsin and serum protease, is brought out by the difference in behavior toward protease inhibitors. Hussey and Northrop (1923), Kunitz and Northrop (1936), and Grob (1943) have shown that the kinetics of inhibition by either serum or pancreatic inhibitor indicates the reaction to be due to the formation of a dissociable, equimolar compound between enzyme and inhibitor. The inhibition of serum protease by these two inhibitors, however, requires much more of either inhibitor in order to inhibit the same amount of proteolytic activity than in the case of trypsin. In addition to this marked quantitative difference, the shape of the inhibition curves of the two enzymes in the presence of either inhibitor differs so markedly as to indicate the possibility of a different mechanism of inhibition. Several explanations might be advanced for these differences in the inhibition of the two enzymes. It is possible that the inhibition of serum protease is not an equimolar reaction, but that several molecules of inhibitor must combine with the enzyme before inhibition is complete. It is also possible that trypsin is more actively proteolytic per molecule than is serum protease, or it is possible that the dissociation of the serum protease-inhibitor complex differs greatly from that of the trypsin-inhibitor complex.

The identical behavior of both the serum and pancreatic inhibitors toward both trypsin and serum protease furnishes additional evidence that the two inhibitors are very similar if not identical, as postulated by Schmitz (1938).

Further evidence as to the non-identity of the two enzymes is shown in the study of their combined effect on a single substrate. The data indicate quite clearly that trypsin is able to continue the hydrolysis of casein after hydrolysis by serum protease is practically complete. In the light of the work of Bergmann (1941, 1942) and his coworkers on the peptide bond specificity of proteases, this may be interpreted as indicating that serum protease and trypsin split different linkages in the substrate molecule. When the converse of this experiment was carried out, that is, when trypsin was allowed to act first, followed by serum protease, no further hydrolysis took place, even though the action of trypsin was not complete, as indicated by further hydrolysis on the addition of more trypsin. In explaining these findings it may be suggested that the linkages split by serum protease are among those split by trypsin and

that the latter enzyme is able to split additional linkages. It is also possible that the linkages split by serum protease are contained in the acid-soluble fragments of trypsin digestion, and under the conditions of the experiment it is not possible to detect the hydrolysis of these bonds by serum protease. In any event, it is apparent that trypsin is able to split linkages in casein which serum protease cannot.

It has not been possible in any experiment so far devised to demonstrate that fibrinolysin affects in any way the inhibition of serum protease or of trypsin by either serum or pancreatic inhibitors.<sup>4</sup> It appears, therefore, that the normal inactive state of serum protease in the serum is not due to the inhibitor also present in the serum, but involves some other mechanism. It is interesting to note that prior to the studies of Kunitz and Northrop the inactivity of pancreatic extracts was explained on the basis that the proteases were combined with the inhibitor also present in the same extracts (Northrop, 1939). Kunitz and Northrop (1936) demonstrated that these enzymes are inactive because they occur in a precursor or zymogen state from which they can be activated by certain specific activators (kinases) and in some cases by non-specific agents such as magnesium and ammonium sulfate. Following activation from the precursor state, the active enzyme may then combine with inhibitor with resulting loss of activity.

While it has not been possible to demonstrate an action of fibrinolysin on serum protease inhibited by either serum or pancreatic trypsin inhibitors, it has been shown that the activation of serum protease from its normal state in serum by fibrinolysin is apparently a catalytic reaction. The kinetics of this reaction, in the absence of large amounts of serum inhibitor, indicates that it is enzymatic in character, as is the kinase activation of the pancreatic proteases.

<sup>4</sup> Mirsky (1944) has recently reported inhibition of the fibrinolytic activity of streptococcal cultures by addition of crystalline trypsin inhibitor of Kunitz and Northrop (1936) and the inhibitor extracted from soybeans by Ham and Sandstedt (1944). Mirsky suggests that inhibition is due to inactivation of fibrinolysin by inhibitors and that fibrinolysin is a protease related to trypsin. The apparent contradiction between the results presented in the present paper and those obtained by Mirsky would appear to be based on differences in the amounts of inhibitor used. A satisfactory method for the measurement of the small amount of proteolytic activity in unconcentrated serum has not been available to us, but based on estimates of the proteolytic activity of unconcentrated serum, and the amount of crystalline trypsin inhibitor necessary to inhibit the serum protease concentrates, it appears that Mirsky used levels of trypsin inhibitor 100-500 times as high as would be necessary to completely inhibit all of the serum protease activity in his fibrinogen preparations. Thus, even though fibrinolysin was added to the mixture, the tremendous excess of inhibitor would result in inactivation of serum protease as rapidly as it was activated. The results obtained by Mirsky, therefore, are probably due to inhibition of the activated protease by an excess of inhibitor rather than to inhibition of fibrinolysin.

The rate of activation is directly proportional to the fibrinolysin concentration and the activation has a temperature coefficient of about 1.8. Following activation by fibrinolysin, the active protease may be inhibited by serum or pancreatic trypsin inhibitor, but as noted above, this inhibition cannot be influenced by fibrinolysin. In the presence of serum inhibitor, the kinetics of serum protease activation is complicated by combination of the activated enzyme with inhibitor, causing the course of the reaction to deviate from that of a first order reaction. Northrop (1939) has shown that the kinetics of activation of pepsinogen and trypsinogen also shows a deviation from the theoretical in the presence of inhibitors of these enzymes.

Serum protease may also be activated by chloroform and other non-specific agents. The mechanism of activation by these agents requires further study. It is possible that one of two or more mechanisms is involved. Bodine and his coworkers (1937, 1938, 1943, 1944) have shown that treatment with certain denaturing agents, such as chloroform or certain detergents, activates the protyrosinase of grasshopper eggs. Activation apparently involves slight denaturation of the proenzyme protein. On the other hand, the function of chloroform in the activation of serum protease may be the removal of serum inhibitor, allowing spontaneous activation of the protease, analogous to the spontaneous activation of trypsinogen which occurs in the absence of trypsin inhibitor. Certain evidence supporting this latter hypothesis is available in the case of serum protease. Teale and Bach (1919) have shown that serum inhibitor can be removed from serum by organic solvents only when coagulation of protein occurs. In this laboratory it has been noted that solutions of inactive serum protease (lysin factor) which contain little or no serum inhibitor may become active spontaneously on standing for several weeks in the refrigerator (Christensen, 1945). It is possible, therefore, that chloroform treatment of serum results in the removal of serum inhibitor by producing protein denaturation. Upon removal of the inhibitor, spontaneous activation of the serum protease may occur. This spontaneous activation may be autocatalytic in nature. The proteolytic enzyme precursors pepsinogen and trypsinogen are autocatalytically activated with great rapidity, which is apparently not true of serum protease. On the other hand, chymotrypsinogen shows some spontaneous activation, but the process is much slower than with pepsinogen and trypsinogen (Kunitz and Northrop, 1935).

The data obtained in the present experiments are compatible with the hypothesis that the proteolytic enzyme system of serum is analogous to the proteolytic enzyme systems of the pancreas, trypsin and chymotrypsin. The serum enzyme occurs normally in serum and plasma in an inactive precursor state, similar to trypsinogen and chymotrypsinogen. Activation from this precursor state can be accomplished by treatment with streptococcal fibrinolysin, which acts in a manner analogous to enterokinase or the

mold kinase of Kunitz (1938). Activation may also be produced by other means, the mechanism of which is not yet understood, but which may involve autocatalytic activation. Following activation, the active enzyme, in common with trypsin, may be inhibited by crystalline pancreatic trypsin inhibitor or an inhibitor present in serum.

Study of the properties of serum protease suggests that a revised terminology should be introduced in order to describe it more adequately. Terms such as "serum trypsin" and "serum tryptase" are unsuitable because they imply a relationship between the serum protease and trypsin. The data recorded in the present paper indicate that these enzymes are dissimilar. The older terms "fibrinolysin" and "serum fibrinolysin" are also unsuitable because the enzyme is active against proteins other than fibrin. "Serum protease" is not completely satisfactory because it is binomial and also because the term "serum proteasogen" to indicate the zymogen form is cumbersome. Since the proteolytic enzyme of serum appears to be analogous to that of certain of the pancreatic proteases, a nomenclature based on the principles employed in designating the latter enzymes is suggested. Under this scheme the activated enzyme may be termed "plasmin" in conformity with common usage for proteases, where the prefix indicates the source of the enzyme, followed by -in, as with "bromelin," "ficin," and "papain." The inactive enzyme as it occurs in serum and plasma may be designated as "plasminogen" to indicate its source, the plasma, and also to indicate that it is in an inactive, precursor state. Streptococcal fibrinolysin, also a misnomer in the light of present knowledge, may be termed "streptokinase," analogous to "enterokinase" or "mold kinase." The term "plasmin" has been used in the past to designate a fraction of blood obtained by a special salting-out procedure. This usage, however, has become obsolete and the possibility of confusion with the proteolytic enzyme system is remote.

#### CONCLUSIONS

1. Fibrinolysin-activated lysin factor and chloroform-activated serum protease of serum and plasma are one and the same enzyme, differing only in their mode of activation.

2. The enzyme as it normally occurs in serum or plasma is not inactive because of combination with serum inhibitor. It is present as an inactive precursor or zymogen and may be activated from this state by streptococcal fibrinolysin.

3. The activation of serum protease by streptococcal fibrinolysin is a catalytic reaction, analogous to the kinase activation of trypsinogen by enterokinase. Treatment of serum or plasma with chloroform apparently results in removal of serum inhibitor which may allow autocatalytic activation of the serum protease.

4. The serum enzyme differs from trypsin in its pH of optimum activity,



in its reactions with specific protease inhibitors, and in its action on casein.

5. A revised nomenclature for the serum enzyme system is suggested which more accurately describes its properties than the terms in current use.

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## STUDIES ON THE pH AND Eh OF NORMAL AND INFLUENZA-INFECTED EGGS\*

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The value of the embryonated egg for investigations of many biological problems is becoming increasingly apparent. For example, it has found application in studies on the production and propagation of numerous human and animal viruses (1-11), the evaluation of various bacteriostatic agents (12-14), the determination of tissue toxicity of newly synthesized compounds (15, 16), and investigations on malignant cell pathology (17-20). Many of the physical and chemical changes accompanying growth of the chick embryo and development of its extra-embryonic membranes and fluids are now known. Eh values in the normal chick embryo (21, 22), pH determinations on normal allantoic fluid (23-25), biometrical studies of embryo length-weight relationships (26-28), and chemical analysis of embryonic tissues (29-31) have been reported. It has been established that the amount of tissue, the pH of the surrounding medium, and the oxidation-reduction potential of the system in which a virus is propagated are factors influential in the maintenance of optimal growth conditions (32-35).

The purpose of this paper is to report investigations of the pH and Eh values of allantoic fluids from fertile eggs infected with influenza virus, and to compare these values with the pH and Eh values obtained using non-infected eggs.

### *Materials and Methods*

*Eggs.*—White Leghorn eggs, received at weekly intervals from a single hatchery, were used throughout this series of experiments. Since there was considerable varia-

\* The opinions advanced in this publication are those of the writers and do not represent the official views of the Navy Department.

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tion in size and weight (36-39), it was decided to use only eggs falling within the range of 53 to 65 gm., which represents a deviation of approximately one sigma from the mean of 208 observations. All eggs, turned twice daily, were incubated at  $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  in a forced-draft electric incubator at a relative humidity of 55 to 60 per cent (40). Fertility ranged from 80 to 85 per cent.

*"Days of Incubation."*—In considering the relationships of physicochemical changes to the incubation period, it is important that the expression "days of incubation" be clearly defined. Marked variations were found in the embryo weights of eggs incubated for identical periods of time. Since the actual age of a given embryo is difficult to establish, the weight of the embryo has been adopted as an index of embryonic maturation. All statements in this paper as to "days of incubation" are therefore based upon the weight of an embryo falling within one sigma of the mean weight of a group of embryos incubated for a known number of days.

*Virus Inoculum.*—The inoculum for all eggs consisted of 0.1 ml. of a  $10^{-8}$  saline dilution of PR8 egg-passage virus having a red blood cell agglutination titer of 1:1280 (41). Undiluted samples of this virus were kept at  $-72^{\circ}\text{C}$ . in individual lusteroid tubes, a separate tube being used each day. In this manner it was possible to avoid titer loss due to repeated freezing and thawing.

*Method of Inoculation.*—The position of the embryo was marked during candling of the eggs. After cleansing of the shell with 1 per cent lysol, a small puncture was made with a sharp dissecting needle both above the air space and through the embryo position outlined on the side. The inoculation of virus was made through the lateral opening into the allantoic cavity by means of a tuberculin syringe and needle. The puncture holes were then sealed with paraffin and the eggs incubated for an additional 48 hours.

*Methods of Harvesting Allantoic Fluids.*—The allantoic fluid was removed in one of two ways, depending upon the age of the embryos:

(a) For the 9 day eggs, following removal of the shell over the air space, the chorion was stripped from the allantoic membrane, exposing the vascular network. With reasonable caution it was then possible to remove the allantoic membrane without excessive bleeding. A small thimble of copper screen, which had been dipped into an acetone-cellulose mixture and dried, was gently lowered into the allantoic cavity. The thimble rapidly filled with the fluid, thus making it possible to remove the maximum amount of allantoic fluid with a glass syringe.

(b) For the older eggs the chorio-allantoic membrane was carefully removed and the allantoic fluid poured into a sterile Petri dish, from which it was introduced into the specimen cup of the instrument. Less than a minute's time was required for the complete operation. Fluids contaminated with yolk, excessive amounts of blood, or amniotic fluid were discarded. The presence of small amounts of red blood cells in the fluids being tested did not alter or affect the accuracy of the measurements materially, as determined by a comparison of bloody fluids before and after centrifugation.

*Method of Measuring Eh and pH.*—The determinations of pH and Eh were made simultaneously by the use of two Beckman Laboratory Model G vacuum-tube electrometers. Roblin and Bell (42) found this type of instrument to be accurate to plus or minus 3 mv., which is acceptable for measurements of biological materials (43). The effect of polarization in this instrument is eliminated since the grid current is only

$10^{-12}$  amperes. The instrument is sensitive to 0.01 pH units with a working accuracy of 0.05 pH units. By using a common lead (44) from the single calomel electrode to the terminals of the two amplifiers, it was possible to use one of the instruments for pH and the other for Eh measurements.

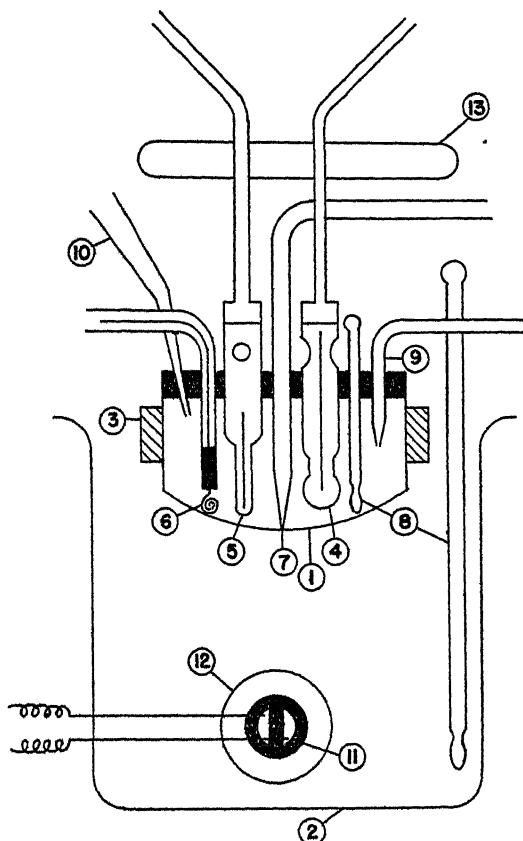


FIG. 1. Schematic diagram of the electrode assembly. (1) lusteroid cup; (2) 500 ml. pyrex beaker; (3) stainless steel holder; (4) glass electrode; (5) saturated calomel electrode; (6) bright platinum electrode; (7) suction; (8) thermometer; (9) distilled water inlet; (10) specimen inlet; (11) double contact bulb; (12) sealed-in pyrex tube; (13) plastic bar.

Fig. 1 (45-52) shows the arrangement of apparatus. The calomel and the glass electrodes were those supplied with the Beckman meters. The calomel half cell is of the saturated KCl type, which at 30°C. has an  $E_c$  value of 0.2420 (53). The glass electrode contains a reference fluid and an inner electrode, a system which is known to us only as formula "Beckman 290" (54). This electrode arrangement simulates the activity of a quinhydrone half cell. The glass membrane is constructed from a mate-

rial similar to Corning 015. The platinum electrode, constructed in this laboratory, consisted of a wire, 7 mm. long, formed into a loop and fused into a pyrex tube with the aid of a soft glass bead. The wire leading from this electrode made contact with the platinum by means of a mercury junction.

External electrostatic forces were prevented from influencing the measurements by housing the electrode assembly within a shield constructed of fibre board and copper screen (49) (Fig. 2). The copper shielding extended into the Beckman electrode chambers by means of "tunnel" extensions (Fig. 3). The entire assembly rested on a grounded galvanized iron base (50). High tension automobile cable leads, suspended by means of plastic insulators, provided an added precaution against internal voltage leakages. The portions of the electrodes protruding from the chamber were coated with paraffin (52).

A liter container suspended on the outside of the housing and leading into the specimen cup provided distilled water for washing the electrodes. A suction line leading to the bottom of the specimen cup enabled removal of the specimen and washings without the necessity of disturbing the electrode assembly. In this manner, it was possible to wash the electrodes 5 to 6 times in less than a minute. The electrodes, the tubes for the wash water and suction, the thermometer, and the specimen inlet tube were sealed into a plastic disc ground to fit a lusteroid tube 3 cm. in diameter. This was immersed in an oil bath maintained at a temperature of 30°C., plus or minus 0.5°C.

The influence of air, nitrogen, and carbon dioxide was investigated by exposing the surface of the liquid specimen to these gases. The CO<sub>2</sub> resulted in a rapid rise in oxidation-reduction potential and a drop in pH due to the accumulating carbonic acid. The subsequent introduction of N<sub>2</sub> gas caused the pH to return to its original value, and the Eh dropped below the equilibrium value as determined under atmospheric conditions. It was therefore felt that a normal atmosphere of air would give results most closely approximating the true conditions of oxidation-reduction existing in the non-infected allantoic fluid.

The instruments were permitted to warm up for an hour before use. Before each series of runs, the meters were calibrated with 0.05 M acid phthalate (pH 3.97) and 0.1 M phosphate buffer (pH 7.0). In practice, observations on each sample were made at 10 minute intervals until less than 4 mv. variation was obtained in successive readings. Immediately following removal of each allantoic fluid sample from the egg, an aliquot was introduced into the specimen cup and measurements made as described above.

#### EXPERIMENTAL

*Embryo, Weight-Age Relationship.*—Each embryo upon removal from the shell was placed on filter paper to remove excess fluid, and then measured from "crown to rump" to the nearest 0.5 mm. Weighings were made to the nearest 10 mg. The values obtained for the weights of non-infected embryos are shown in Table Ia and are plotted in Fig. 4. Similar data recently published by Walker (23) are included for comparison. The observation of Lerner and Gunns (37) that total egg weight bears no relation to the weight of the developing embryo was confirmed by the results of routine weighing of all eggs prior to incubation (Table II).

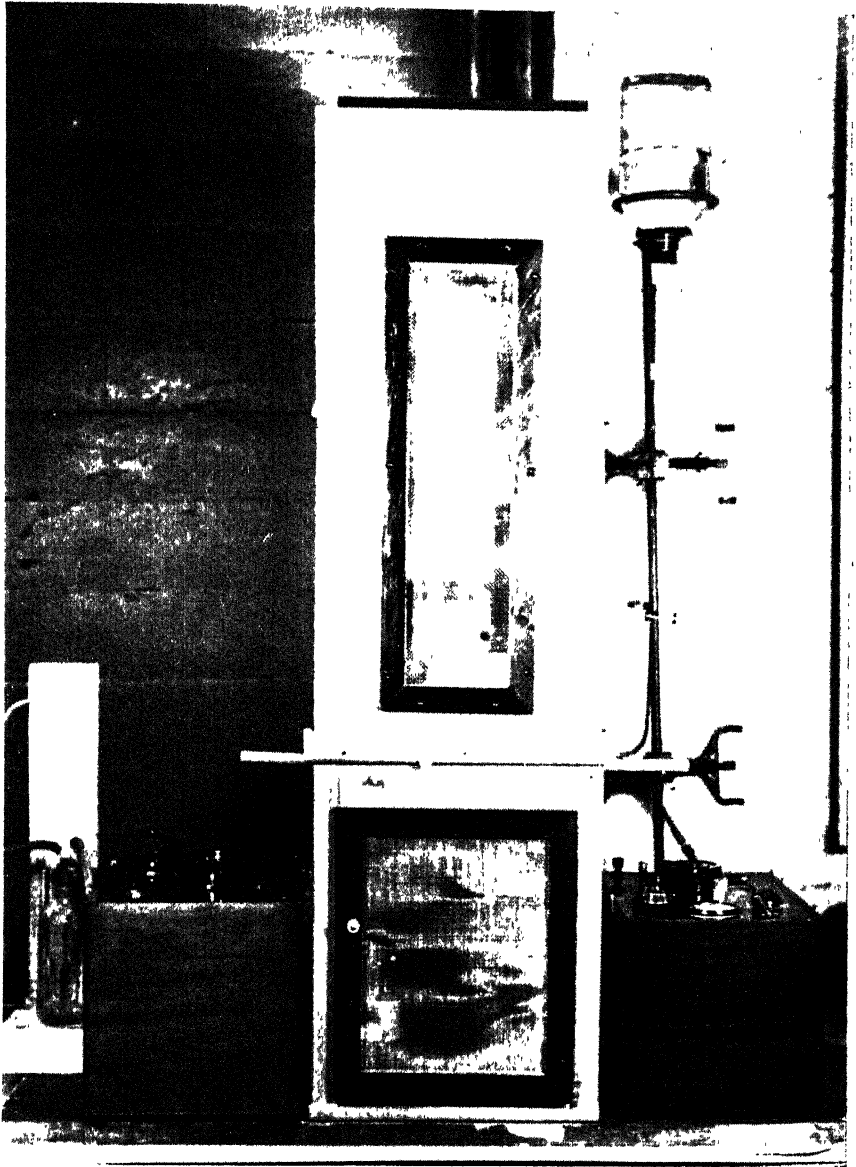


FIG. 2. Apparatus used for determining Eh and pH of allantoic fluids.

In addition it was found that embryos from infected eggs weighed consistently less than non-infected embryos. This reduction in development was equivalent to approximately 12 hours' normal growth. From an inspection of the results



for infected and non-infected embryo weights (Fig. 5) it appears that the proportional decrease in weight as a result of virus infection was relatively constant throughout the period tested. (See Table Ib.)

*pH Values of Non-Infected Allantoic Fluids.*—The values obtained for non-infected allantoic fluids from eggs of different ages are shown in Table IIIa and graphically illustrated in Fig. 6. For purposes of comparison, Walker's (23) and Yamada's (24) data are also included. The middle portions of the curves

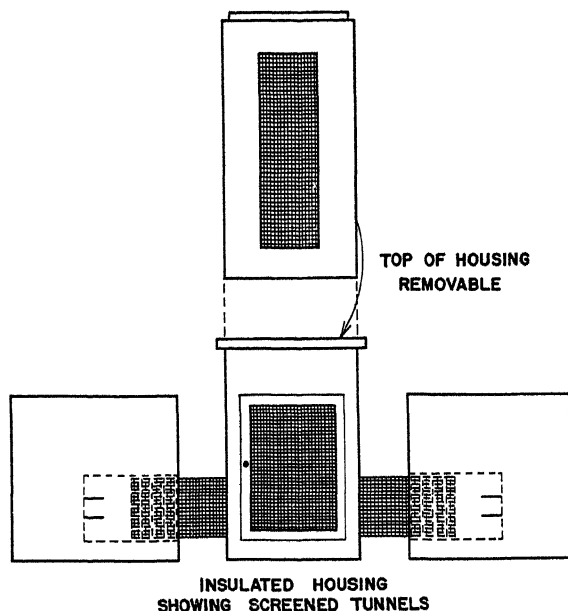


FIG. 3. Insulated housing showing screened tunnels.

show a striking similarity in slope; our data, however, show consistently higher values for each day. Such differences may be accounted for by variations in eggs, apparatus, and technics used. The relatively constant pH of young eggs, 9 to 12 days old, is clearly brought out in this series of observations. From this period on through the 17th day, the drop in pH was rapid and consistent. There are abrupt deviations from the curve appearing on the 11th and the 18th day, followed by a return to the previous slope at the 12th and 19th days. A similar observation is apparent in Walker's (28) findings for this period of development. An analysis of variance (55) calculated on these data (Table IIIb) confirms the obvious impression of the graph that the pH of non-infected allantoic fluids is a function of the age of the embryo.

*Eh Values of Non-Infected Allantoic Fluids.*—Eh is the potential of an indiffer-

TABLE Ia  
*Embryo Weights of Non-Infected Eggs*

Time of incubation	No. of observations	Weight range	Mean weight	Mean weight (Walker (23))
<i>days</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
9	20	1.28- 1.58	1.38	1.44
10	26	1.68- 2.12	1.90	1.88
11	21	2.35- 3.03	2.69	2.89
12	20	3.52- 4.58	4.05	3.65
13	30	4.91- 6.11	5.46	5.41
14	39	6.14- 8.36	7.25	7.72
15	12	9.32-11.27	10.29	10.69
16	16	10.68-14.96	12.82	12.67
17	10	14.25-15.11	14.93	15.22
18	7	16.64-18.80	17.72	17.58
19	7	22.28-27.48	24.88	21.13

TABLE Ib  
*Embryo Weights of Infected Eggs*

Time of incubation	No. of observations	Weight range	Mean weight
<i>days</i>		<i>gm.</i>	<i>gm.</i>
11	10	2.21- 2.84	2.49
12	16	3.00- 4.07	3.45
13	11	3.14- 5.13	4.35
14	14	5.40- 7.42	6.33
15	8	7.92- 9.18	8.41
16	14	9.21-13.45	10.64
17	8	12.21-15.55	13.94

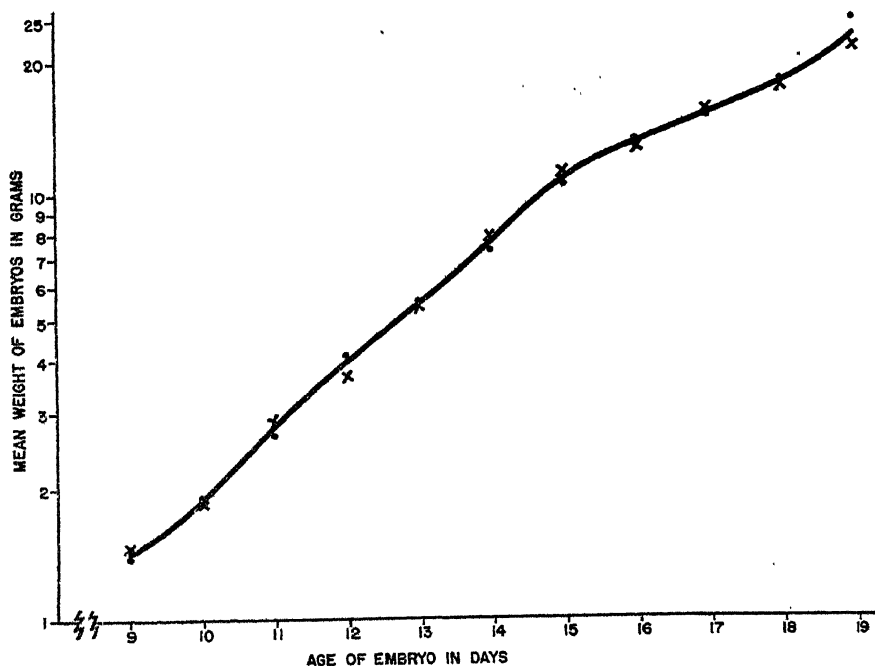


TABLE II

*Comparison of Embryo Weights from Jumbo and Experimental Eggs*

Eggs*	Weight range	Mean weight	Time of incubation	No. of observations	Embryo weight
	gm.	gm.	days		gm.
Experimental.....	53-63	57.6	10	26	1.90
			14	39	7.25
Jumbo.....	69-82	75.9	10	5	1.96
			14	5	7.71

\* Eggs were weighed prior to incubation.

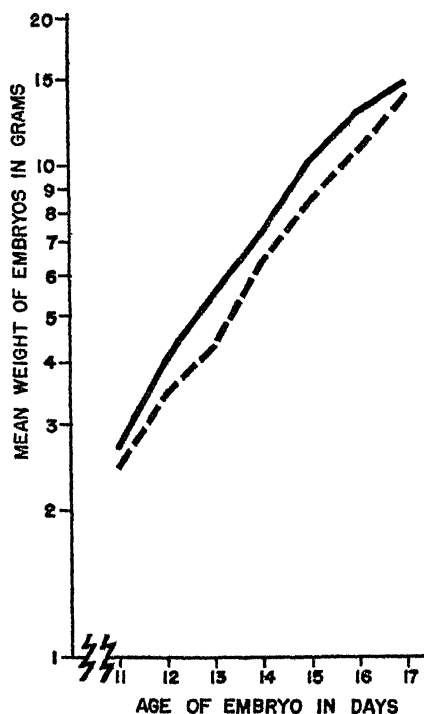


FIG. 5. Age-weight relationship of non-infected and infected embryos. ----, infected series; —, non-infected series.

ent electrode referred to that of the standard hydrogen electrode,  $E_0$ , and is a measure of intensity of oxidation-reduction rather than an expression of quantity. Throughout this report the intensity of the oxidation-reduction potential

TABLE III<sub>a</sub>  
*pH of Non-Infected Allantoic Fluid*

Time of incubation	No. of observations	pH range	Mean pH	$\sigma^*$	Mean pH (Walker (23))	Mean pH (Yamada (24))
<i>days</i>						
9	16	7.16-8.18	7.86	0.432	7.53	7.68 (8 day)
10	23	7.24-8.13	7.85	0.248	7.49	7.33
11	16	7.22-8.12	7.70	0.334	7.31	
12	19	7.45-7.96	7.71	0.337	7.39	
13	25	6.00-8.19	7.53	0.407	7.39	7.41
14	33	5.84-7.92	7.25	0.565	6.79	
15	10	5.61-7.63	6.86	0.630	5.87	5.70
16	16	5.31-7.40	6.19	0.534	5.75	5.67
17	10	4.99-6.35	5.64	0.534	5.60	5.72
18	7	4.88-6.38	6.17	0.534	5.71	
19	7	4.75-6.29	5.56	0.238	5.55	

\* Standard deviation calculated according to the formula for small samples.

TABLE III<sub>b</sub>  
*Analysis of Variance: pH of Non-Infected Allantoic Fluids*

Time of incubation	No. of observations	Sum of observations	Mean of observations	
<i>days</i>				
9	16	125.85	7.86	
10	23	180.65	7.85	
11	16	123.24	7.70	
12	19	146.60	7.71	
13	25	188.34	7.53	
14	33	238.40	7.25	
15	10	68.65	6.86	
16	16	99.04	6.19	
17	10	56.43	5.64	
18	7	43.20	6.17	
19	8	44.52	5.56	
Total.....	183	1,314.95	7.18	
Source of variation	Degrees of freedom	Sum of squares	Mean squares	F*
Total.....	182	137.62	0.756	
Between means of days.....	10	99.78	9.978	F = 45.31
Within days.....	172	37.84	0.220	

\* F = ratio between two independent and unbiased estimates of the variance of a variable which is normally distributed (63).

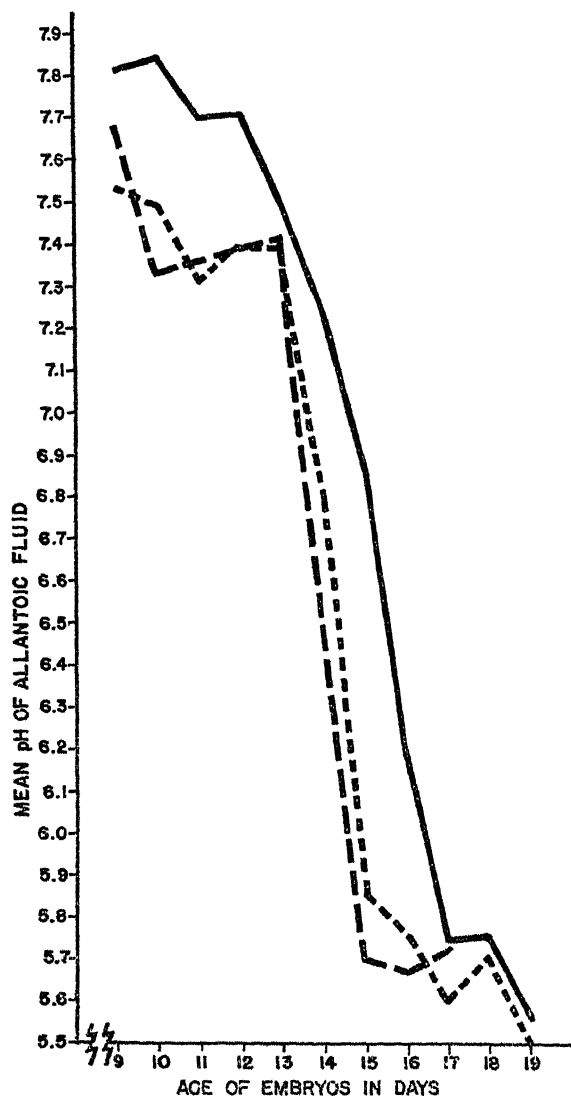


FIG. 6. Comparison of data on pH-age relationship. —, data from Table IIIa; ---, Walker's data (23); — · —, Yamada's data (24).

of the system under investigation will be indicated by "Eh."<sup>1</sup> Since the apparatus employed in our determinations utilized a calomel half cell with a potential

<sup>1</sup> In 1923 Clark and Cohen (56) suggested the use of the expression "redox potential," or "rH," defined as the logarithm of the reciprocal of the hydrogen pressure. In terms of rH and pH, Eh (at 30°C.) is equivalent to 0.03 (rH-2pH). This equation presumes

TABLE IVa  
*Eh Values for Non-Infected Allantoic Fluids*

Time of incubation	No. of observations	Eh range	Mean Eh	$\sigma^*$
<i>days</i>		<i>volts</i>	<i>volts</i>	
9	15	+0.250 to +0.325	+0.292	0.042
10	20	-0.271 to +0.270	-0.003	0.274
11	13	+0.251 to +0.301	+0.268	0.023
12	17	-0.271 to +0.274	-0.051	0.268
13	25	-0.286 to +0.311	+0.083	0.105
14	29	+0.252 to +0.322	+0.285	0.284
15	9	+0.243 to +0.282	+0.267	0.025
16	15	+0.262 to +0.334	+0.307	0.025
17	10	+0.252 to +0.334	+0.312	0.026

\*  $\sigma$  calculated according to formula for small samples.

TABLE IVb  
*Analysis of Variance: Eh of Non-Infected Allantoic Fluids*

Time of incubation	No. of observations	Sum of observations	Mean of observations	
<i>days</i>				
9	15	4.38	0.292	
10	20	0.06	0.003	
11	13	3.49	0.268	
12	17	0.86	0.051	
13	25	2.09	0.083	
14	29	8.29	0.285	
15	9	2.41	0.267	
16	15	4.61	0.307	
17	10	3.12	0.312	
Total.....	153	29.31	0.179	
Source of variation	Degrees of freedom	Sum of squares	Mean squares	F*
Total.....	152	6.50	0.0427	
Between means of days .....	8	2.90	0.3634	F = 14.52
Within means.....	144	3.60	0.0250	

\* F = ratio between two independent and unbiased estimates of the variance of a variable which is normally distributed (63).

a direct relationship between the oxidation-reduction potential, the hydrogen ion concentration, and the hydrogen pressure. However, since biological fluids are usually well buffered, and since alterations in Eh due to the pH in these systems are negligible (43), the term "redox potential" is to be avoided (57).

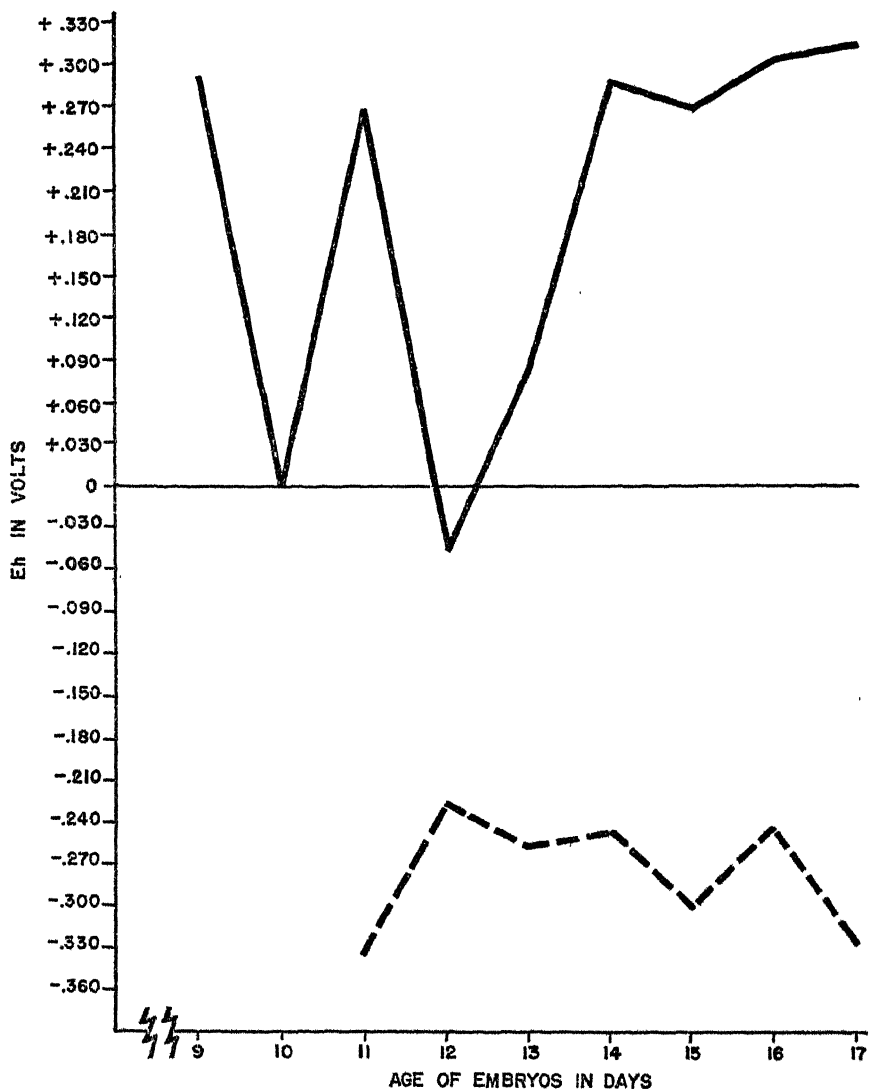


FIG. 7. Eh-age relationship of non-infected and infected allantoic fluids. —, non-infected series; ---, infected series.

( $E_c$ ) of  $\pm 0.2420$  mv., the voltage reading of the potentiometer ( $E$ ) was corrected by this figure in order to find the true  $E_h$  value:

$$E_h = E \pm 0.2420.$$

The values for the  $E_h$  of certain portions of the chick embryo have already been determined. Friedheim (21) studied the oxidation-reduction potential of

TABLE Va  
*pH Values for Infected Allantoic Fluids*

Time of incubation	No. of observations	pH range	Mean pH	$\sigma^*$
<i>days</i>				
11	10	6.94-7.51	7.27	0.347
12	16	7.34-7.83	7.54	0.229
13	11	7.11-7.85	7.52	0.406
14	14	6.81-7.78	7.38	0.290
15	8	7.09-7.36	7.25	0.381
16	15	6.50-7.65	7.07	0.387
17	9	6.45-6.99	6.82	0.378

\*  $\sigma$  calculated according to formula for small samples.

TABLE Vb  
*Analysis of Variance: pH of Infected Allantoic Fluids*

Time of incubation	No. of observations	Sum of observations	Mean of observations	
<i>days</i>				
11	10	72.76	7.27	
12	16	120.66	7.54	
13	11	82.81	7.52	
14	14	103.34	7.38	
15	8	58.07	7.25	
16	15	106.11	7.07	
17	9	61.45	6.82	
Total.....	83	605.20	7.29	
Source of variation	Degrees of freedom	Sum of squares	Mean of squares	F*
Total.....	82	10	0.12	
Between means of days.....	6	4	0.66	F = 1.19
Within means of days.....	76	6	0.79	

\* F = ratio between two independent and unbiased estimates of the variance of a variable which is normally distributed (63).

chick thymus macerates, using Michaelis' mercury electrode. Pavlov and Issakawa-Keo (22), using the platinum electrode, investigated the Eh of the avian yolk and white.

The Eh values found for non-infected allantoic fluids in our egg series are shown in Table IVa and are plotted in Fig. 7. Although there is overlapping



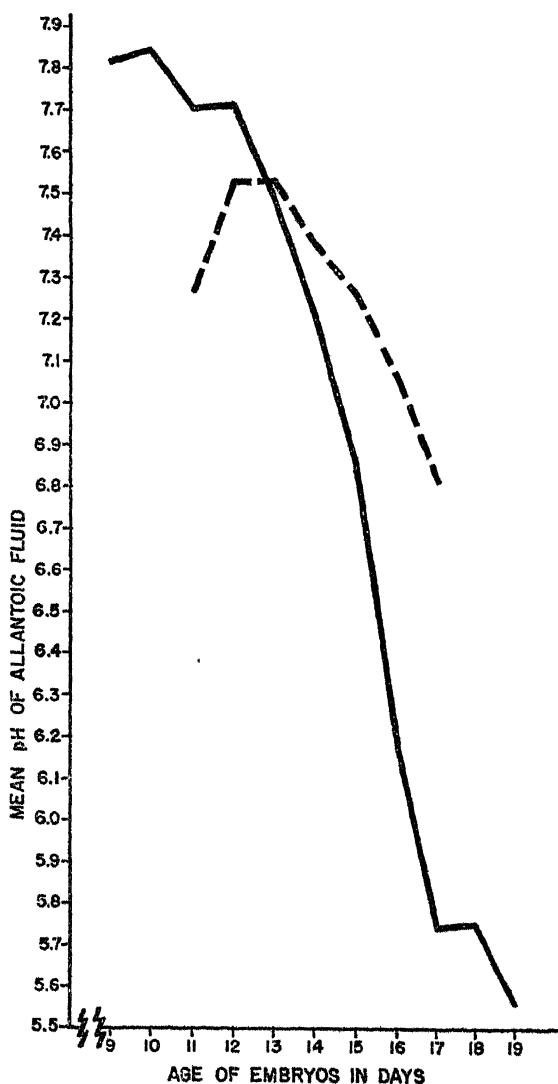


FIG. 8. pH-age relationship of non-infected and infected allantoic fluids; —, non-infected series; ---, infected series.

of individual values among the days of incubation, statistical treatment of the data by the method of analysis of variance (Table IVb), indicates that the means differ significantly among themselves; *i.e.*, the means vary more than would ordinarily be expected if they were drawn at random from a homogeneous

population of eggs. Therefore, there is some indication that Eh of non-infected allantoic fluids is dependent upon the age of the embryo.

TABLE VIa  
*Eh Values of Infected Allantoic Fluids*

Time of incubation	No. of observations	Eh range	Mean Eh	$\sigma^*$
<i>days</i>		<i>volts</i>	<i>volts</i>	
11	10	-0.288--0.382	-0.333	0.054
12	16	+0.296--0.382	-0.217	0.240
13	11	+0.312--0.366	-0.255	0.174
14	14	+0.342--0.420	-0.245	0.174
15	8	-0.261--0.387	-0.300	0.029
16	15	+0.334--0.371	-0.244	0.243
17	9	-0.277--0.372	-0.325	0.027

\*  $\sigma$  calculated according to formula for small samples.

TABLE VIb  
*Analysis of Variance: Eh of Infected Allantoic Fluids*

Time of incubation	No. of observations	Sum of observations	Mean of observations
<i>days</i>			
11	10	-3.33	-0.333
12	16	-3.48	-0.217
13	14	-3.57	-0.255
14	14	-3.47	-0.247
15	8	-2.40	-0.300
16	15	-3.67	-0.244
17	9	-2.93	-0.325
Total. ....	86	-22.85	-0.265

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F*
Total. ....	85	2.85	0.0335	F = 2.09
Between means of days. ....	6	0.10	0.0166	
Within means. ....	79	2.75	0.0347	

\* F = ratio between two independent and unbiased estimates of the variance of a variable which is normally distributed (63).

*pH Values of Infected Allantoic Fluids.*—The experimental data for the pH values of infected allantoic fluids are shown in Table Va and illustrated in Fig. 8. The variation between means of different age eggs in this series is considerably less than in the non-infected group. Analysis of variance of the data reveals that variation between individual eggs is such that the differences between

pH at the various ages do not appear significant (Table Vb). In other words, the pH of allantoic fluids of eggs 48 hours after infection is more a function of the presence of the virus in the egg than of the age of the embryo.

*Eh Values of Infected Allantoic Fluids.*—The Eh values obtained from infected allantoic fluids indicate the existence of a state of marked reduction (Table VIa). This is similar to the reduced state of the potential usually found in bacterial cultures (43). The wide range of values within any 1 day is due to the occasional Eh on the positive side; however, the mean values for all days are definitely of a negative potential.

#### DISCUSSION

It is evident that for the first half of the embryonic development the pH range is on the alkaline side just above neutrality. After the 14th day there is a consistent decrease of the pH to a mean of 5.56 on the 19th day. Needham (26) has offered chemical data which logically explain this increasing acidity. He quotes workers who have shown that as the embryo develops there is an increase in the uric acid level, a reduction of the calcium and phosphate buffer salts, and an increase in the  $\text{CO}_2$  tension and  $\text{H}_2\text{CO}_3$  content. All these factors tend to lower the pH of the allantoic fluid.

The determination of the Eh in biological systems is as yet difficult. We found it necessary to take a number of readings on each specimen before the potential reached equilibrium. Conant (58) found the same situation to exist in methemoglobin systems. Gortner (59) points out that many oxidation-reduction potentials are not stable and achieve equilibrium very slowly.

From Fig. 7 it can be seen that there is no consistent and definite trend as to the direction of the potential in the non-infected allantoic fluids of the chick embryo throughout the series tested. However, from the 14th day on, the potential remains strongly positive. Statistical analysis of the data obtained for the values of the Eh for each day indicates that the differences between the means are greater than could be expected by chance variation, and it must be concluded that the oxidation-reduction potential of the allantoic fluid is definitely correlated with the age of the embryo.

On the other hand, examination of the results obtained for the Eh of the infected allantoic fluids reveals a markedly altered situation. The mean Eh for each day examined exhibits a definitely lowered potential (Table VIb). The analysis of variance calculated for these data shows a lack of significant differences between the mean values for the days of incubation. The value of F indicates that the variation between the means of the various days is within the range of a sample drawn from a homogeneous population. It appears, therefore, that the establishment of an influenza virus infection within the egg consistently brings about a reduction of potential in the allantoic fluid regardless of embryonic age. The standard error of the difference of means between the

Eh values of non-infected fluids and those of the infected fluids shows that such a difference would occur by chance approximately but once in 370 times.

The influence of the electromotive potential of the medium on the growth of viruses and rickettsiae has given rise to many interesting experimental approaches to the problem of increased titer of a virus *in vitro* by including substances with a high negative potential, such as cysteine, arginine, and sodium sulfite (60-62). It is possible that the incorporation of reducing agents in the embryonated egg may result in increased virus production. This might perhaps be achieved by using a reductant combined with the virus inoculum, or by the introduction of a selected reducing agent into the egg at intervals during the growth of the virus.

#### SUMMARY

1. pH values of allantoic fluids from non-infected eggs showed a rapid drop from the 12th to the 17th day of embryonic development. A definite age-pH relationship was shown to exist.

2. The allantoic fluid of eggs infected with influenza virus, contrasting with the non-infected eggs, revealed pH values which remained relatively stable about the neutral point at any age between 11 and 17 days. No relationship between pH and age could be established, in the infected series.

3. The Eh value of the fluid from non-infected eggs also exhibited a significant relationship to embryonic age. The majority of the determinations revealed a positive potential.

4. Similar determination on influenza-infected eggs gave results of a predominantly negative potential, indicating a marked state of reduction accompanying virus multiplication. As with the pH determinations, the Eh values of infected eggs were not a function of their age.

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# THE RHEOLOGY OF THE BLOOD. V\*†

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## *The Fluidity of Egg Albumin*

In our earlier discussion (1) of the fluidity of solutions of the proteins of the blood, fluidity-concentration coefficients for the temperature of the body (37°) were calculated on the assumption that the fluidity is a linear function of the volume concentration of the protein. It was recognized that there might be exceptions to the law and we mentioned the possible case of egg albumin measured by Chick and Lubrznska (2); we attempted, by the use of the original data of Loeb (3) to prove that gelatin is not an exception to the law. We are now convinced that the heading of the original data was incorrect as " $\eta/\eta_0$ " and that " $\log \eta/\eta_0$ " is correct as given later in Proteins and the theory of colloidal behavior. Although gelatin is not important in itself to our study of the blood proteins, it was considered because of measurements over a considerable range of both concentration and temperature. Since we obtained surprisingly simple relations, which no longer hold, we desire now to reconsider gelatin along with egg albumin and a few others. Since egg albumin is not an exception, it will be considered first.

The fluidities of egg albumin given in Table I are for each concentration and temperature used by the authors (2, p. 62); the fluidities of water used for converting relative viscosities to rhes are the values of Bingham and Jackson (4). Since the temperatures are not the same for all of the concentrations, the data are not easily comparable. We have, however, fitted the hyperbolic formula (5)

$$T = A(\varphi + D) - B/(\varphi + D) + C \quad (1)$$

to the fluidity-temperature data,  $A$ ,  $B$ ,  $C$ , and  $D$  being arbitrary constants and  $T$  the temperature Centigrade. The values of these constants are given in Table II. With this equation and the appropriate constants, the values of the

\* Bingham, E. C., and Roepke, R. R., The rheology of the blood, unpublished paper presented before the Society of Rheology; The rheology of the blood. II. The effect of fibrinogen on the fluidity of blood plasma, *J. Am. Chem. Soc.*, 1942, **64**, 1204; The rheology of the blood. III, *J. Gen. Physiol.*, 1944, **28**, 79; The rheology of the blood. IV. The fluidity of whole blood at 37°C., *J. Gen. Physiol.*, 1944, **28**, 131.

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fluidity in rhes have been computed for each 5° from 0–40° and these are given in Table III. These “observed” values are plotted in Fig. 1.

It would be difficult to draw smooth curves through all of the observed points and a little consideration shows why that is or may be unnecessary. In the

TABLE I  
*Fluidity of Egg Albumin*

After Chick and Lubrznska (3).

Protein per cent by weight	Temperature $T$	Fluidity Pure water $\Phi_1$	Fluidity protein $\Phi_{obs.}$
7.04	2.8	61.3	44.8
	8.3	72.8	53.5
	15.2	88.2	63.9
	25.0	111.9	82.3
	32.3	131.0	97.8
	42.1	158.5	121.0
14.6	2.8	61.4	28.4
	8.6	73.4	34.5
	14.7	87.0	41.6
	15.1	87.9	41.9
	25.0	111.9	54.8
	33.1	133.2	66.6
	42.9	160.8	82.9
20.1	0	55.8	13.8
	8.0	72.2	18.6
	17.0	92.4	25.1
	25.4	112.9	31.5
	33.0	132.9	39.4
	41.6	157.0	48.0
28.15	0.6	57.0	4.23
	7.8	71.7	5.91
	15.6	89.1	8.07
	25.4	112.9	11.28
	33.9	135.4	14.83
	41.9	157.9	18.09

first place, the data for pure water do not appear to lie on the extrapolated curves at either the highest or lowest temperatures, in spite of the fact that those data have been used in computing the fluidities of the solutions. This is not peculiar to egg albumin but is a disconcerting fact repeatedly observed in converting relative values to absolute. The clue seems to be that the measurements were made with the Ostwald viscometer, in the use of which it is not customary to make any kinetic energy correction. This is in spite of the fact

that when calibrated with water, say at 25°, the viscometer will not give values for other pure liquids, such as acetone and ether,<sup>1</sup> which are generally accepted;

TABLE II  
*Constants for Equation (1) for Water and Egg Albumin Solutions*

Protein solution weight per cent	A	B	C	D	B/D
0	0.23275	8676.8	35.17	120	72.3
7.04	0.2754	3075.7	17.97	36.86	33.4
14.6	0.2426	7842	55.17	73.37	106.8
20.1	0.8478	346.2	3.97	4.20	82.5
28.15	2.256	51.36	4.52	0.248	210.4

TABLE III  
*The Fluidities of Solutions of Egg Albumin (Crystallized) at Various Temperatures and Concentrations, Both Observed and Calculated by the Formula,  $\Phi = \Phi_1 - \beta b$*

T	$\beta_1$	$\Phi_1$	$\Phi$ 7.04 per cent	$\Phi$ 14.6 per cent	$\Phi$ 20.1 per cent	Remark	Difference <i>per cent</i>
0	1.967	55.8	41.1	25.7	13.8	Obs.	+6.7
			42.0	27.1	16.3	Calc.	
5	2.320	65.8	47.8	30.4	16.6	Obs.	+5.7
			49.5	31.9	19.2	Calc.	
10	2.697	76.5	55.2	36.0	19.9	Obs.	+4.3
			57.5	37.1	22.3	Calc.	
15	3.092	87.7	62.0	41.8	23.5	Obs.	+3.3
			65.9	42.6	25.6	Calc.	
20	3.508	99.5	72.5	48.0	27.6	Obs.	+0.4
			74.8	48.3	29.0	Calc.	
25	3.945	111.9	82.3	54.8	31.9	Obs.	-0.0
			84.1	54.3	32.6	Calc.	
30	4.404	124.9	92.9	61.9	36.5	Obs.	-2.0
			93.9	60.6	36.4	Calc.	
35	4.880	138.4	104.1	69.6	41.4	Obs.	-3.8
			104.0	67.2	40.3	Calc.	
40	5.374	152.4	116.1	77.8	46.4	Obs.	-5.3
			114.6	73.9	44.4	Calc.	

or even for water at temperatures differing considerably from 25°, the temperature of calibration. The formula usually employed for the Ostwald viscometer is, in effect

$$\eta_0 = C_0 \rho t \quad (2)$$

<sup>1</sup> Errors as great as 14 per cent have been noted.

instead of the more exact formula

$$\eta = C_p t - C' \rho / t \quad (3)$$

where  $C_0$  is the constant of the Ostwald viscometer in place of the more exact  $C$ ,

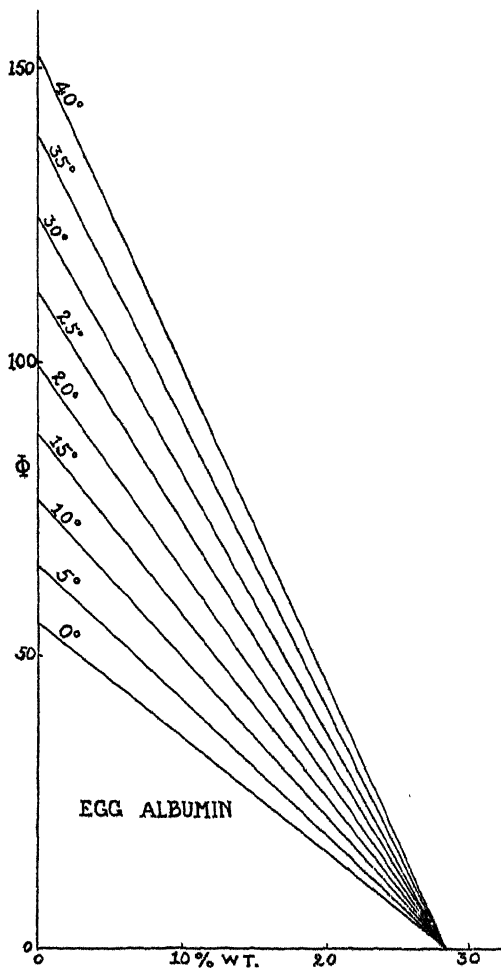


FIG. 1. Fluidity of solutions of egg albumin (crystalline) at various concentrations and temperatures (after Chick and Lubrznska).

and  $C'$  is the constant of the kinetic energy correction,  $p$  is the pressure head, and  $t$  the time of flow of the liquid of density  $\rho$ .

In calibration, the viscosity of water at  $25^\circ$  of 0.894 centipoise (cp.), for example, must be the same irrespective of the formula, hence (6)

$$\eta = C_0 p t = C_p t - C' \rho / t$$

which gives the relation between the two constants  $C_0$  and  $C$  to be

$$C - C_0 = C'_p/p^2 \quad (4);$$

but the relation holds true only at the temperature of 25° for water or for a temperature where the viscosity is the same for other liquids. The error in viscosity ( $\eta - \eta_0$ ) made by the use of the Ostwald formula is

$$\eta - \eta_0 = (C - C_0)pt - C'_p/t \quad (5)$$

This means that the viscosity as calculated by the Ostwald formula is too large above the temperature of calibration and too small below it.

The point here being considered is so important that it seems desirable to give a few illustrative data for water at 0°, 25°, and 40°. The value of  $C'$  (6) is 0.0446  $V/L$  where  $V$  is the volume of flow and  $L$  is the length of the capillary. Assuming a volume of 5 cm.<sup>3</sup> and a length of 10 cm.,  $C' = 0.0223$ . The percentages of error with a pressure head of 10 gm. per cm.<sup>2</sup> are seen to be -3.5 per

TABLE IV

*Illustrations of the Error in Viscometry Caused by the Non-Employment of the Kinetic Energy Correction*

Temperature	Time of efflux	$C p t$	$C'_p/t$	$\eta$	$\eta_0$	Error
°C.		<i>cp.</i>	<i>cp.</i>	<i>cp.</i>	<i>cp.</i>	<i>per cent</i>
0	95.6	1.81	0.02	1.79	1.72	-4
25	47.8	0.94	0.05	0.89	0.89	0
40	37.9	0.75	0.06	0.69	0.705	+2

cent at 0° and +2.4 per cent at 40°. The values of the time of efflux at 0° and 40° given in Table IV have been calculated by means of equation (3). We therefore reach the conclusion that it would be unjustifiable to include the values of the fluidity of pure water in drawing the family of curves from the data available.

Likewise, experience with the flow of suspensions (7) shows that when the concentration is so great that the apparent fluidity approaches zero, the values are often unreliable. It would naturally be supposed that the flow would be too small due to a tendency to clog the capillary, but actually the flow may be greater than expected. The effect may be due to faulty drainage with a tendency to channeling, it may be due to agitation of the bath by the stirrer, or even in some cases to a settling effect. Such effects might explain irregular results but regular effects of this sort are evidence of a yield value in plastic flow (8). We therefore are eliminating from our consideration, at least temporarily, all of the data for the 28.15 per cent mixture on the supposition that it may be plastic and that those data should be considered in a different connection. Using the three remaining concentrations, linear curves are drawn through the points for each temperature, when it is found that all of the iso-

therms meet at approximately the same point on the concentration axis where the fluidity is zero. It is then possible to represent the fluidities on any isotherm by the equation

$$\Phi = \alpha - \beta b \quad (6)$$

$b$  is the weight; it is unnecessary for our purpose here to convert to volume concentration of albumin,  $\alpha$  is obviously the "calculated" value of water, and  $\beta$  is the lowering of the fluidity for each per cent of albumin at the particular temperature. The values of these constants are given in Table V. When  $\Phi = 0$ ,  $b' = \alpha/\beta$ , which is the calculated concentration at which plastic flow should begin and when viscous flow ends; the average value obtained is 28.36 per cent, the value rising slightly with the temperature. The value of  $\beta$  in-

TABLE V  
*The Constants of Equation (2) and  $b' = \alpha/\beta$ , for Egg Albumin*

$T$	$\beta$	$\alpha$	$b'$
0	2.073	55.7	26.8
5	2.359	64.4	27.3
10	2.648	73.9	27.9
15	2.847	82.0	28.8
20	3.370	96.3	28.6
25	3.826	109.2	28.5
30	4.239	122.7	28.9
35	4.719	137.4	29.1
40	5.237	153.0	29.2

creases regularly with the temperature, being in fact  $\Phi_1/b'$ ,  $b'$  being the concentration where the fluidity becomes zero.

It thus appears that the fluidity of egg albumin solutions at concentrations below about 20 per cent and at various temperatures can be calculated from the fluidity of water if we know  $\beta$ . The final form of equation (6) is

$$\Phi = \Phi_1 - \beta b \quad (7)$$

where  $\Phi_1$  is the fluidity of water and  $\beta = \Phi_1/b'$ . Both the observed and the calculated values of the fluidity are given in Table III. At temperatures below 25° the observed values are all below the calculated values, the average percentage differences appearing in the final column. Beyond 25° all but one of the observed values are greater than the calculated values. These facts are qualitatively in accordance with the argument put forward in connection with Table IV. Without the exact dimensions of the viscometer an attempt to make the quantitative corrections is inadvisable, but further precise measurements would present several points of interest.

*The Fluidity of Gelatin*

We are indebted to Dr. David L. Hitchcock, who assisted Dr. Loeb in the revision of *Proteins and the theory of colloidal behavior*, for very helpful information. He says, "In his (Loeb's) original publication [3], *J. Gen. Physiol.*, 1922, 4, 84, the data were labelled  $\eta/\eta_0$ , but I believe that this was a misprint; they should have been marked  $\log \eta/\eta_0$ , as in his book." He also pointed out that Dr. Kunitz assisted Dr. Loeb in making these measurements. In answer to our letter in reference to the matter, Dr. Kunitz makes the very definite statement, "I checked Dr. Loeb's original records on viscosity of gelatin and found that the data for  $\eta/\eta_0 - 1$  at 25°C. are correct as given in Table XL, page 203, of Loeb's *Theory of colloidal behavior* (first edition). The heading 'log  $\eta/\eta_0$ ' is OK for Table XLI, page 204, and hence the heading on page 84 of the original paper is wrong." He appends the further useful statement, "The data for the ordinates of Figs. 6, 11, 12 have been multiplied by 100 for convenience in plotting; something to that effect should have been stated on the figures."

In view of the large number of proteins which have been found to follow the additive law,  $\Phi = \alpha - \beta b$ , it is of importance to study a case which is definitely exceptional. Therefore we are justified in satisfying both ourselves and our readers that gelatin is such an exception and, if possible why it is exceptional. It is quite clear that Loeb himself believed that the logarithms of the viscosity were additive, for he says on page 203, "The formula of Arrhenius leads to a fair agreement." That formula may be written

$$\log \eta - \log \eta_1 = kb \quad (8)$$

or

$$\log \Phi = \log \Phi_1 - kb \quad (9)$$

Using the values of Bingham and Jackson for the fluidity of pure water, the logarithmic fluidities have been computed from the relation

$$\log \Phi = \log \Phi_1 - \log \eta/\eta_1$$

These values are given in Tables VI to X, column 2, and they are plotted in Fig. 2. The logarithmic fluidities at 35°, 45°, and 60° of gelatin certainly indicate a linear relationship, except that: (1) the value for 3 per cent at 60° is off, possibly a typographical error "0.3094" for 0.3594; (2) the curves do not include the values of pure water; and (3) the values for 25° are not linear, the point for 3 per cent being entirely off the figure. Constants have been determined for the linear equation

$$\log \Phi = \alpha - \beta b \quad (10)$$

which are given in Table X. The values of the fluidity calculated by this equation appear in the third column of Tables VI to X, with the percentage

deviation in the fourth column. The average deviation for the solutions above 25° is a little under 0.5 per cent.

Not only are the curves above 25° nearly linear, they are nearly but not quite parallel. The point of intersection of the curves is found by assuming

TABLE VI  
*Fluidity of Solutions of Isoelectric Gelatin*

After Loeb.

Concentration	Log $\Phi_{25}^{\circ}$ observed	Log $\Phi_{25}^{\circ}$ calculated equation (10)	Deviation	$\Phi_{25}^{\circ}$	Log $\Phi_{25}^{\circ}$ calculated equation (11)
<i>vol. per cent</i>			<i>per cent</i>		
0.0	2.0489	2.0511	0.1	111.9	2.0489
0.25	2.0114	2.0092	-0.1	102.6	2.0073
0.50	1.9697	1.9672	-0.1	93.3	1.9697
1.0	1.8807	1.8832	0.1	76.0	1.8825
1.5	1.7724	1.7993	1.5	59.2	1.7993
2.0	1.6788	1.7153	2.2	47.7	1.7161
2.5	1.5798	1.6313	3.4	38.0	1.6329
3.0	1.3548	1.5474	14.2	22.6	1.5497

TABLE VII  
*Fluidity of Solutions of Isoelectric Gelatin*

After Loeb.

Concentration	Log $\Phi_{35}^{\circ}$ observed equation (9)	Log $\Phi_{35}^{\circ}$ calculated equation (10)	Deviation	$\Phi_{35}^{\circ}$	Log $\Phi_{35}^{\circ}$ calculated equation (11)
<i>vol. per cent</i>			<i>per cent</i>		
0.0	(2.1412)	2.1496	+0.4	141.2	2.1412
0.25	2.1143	2.1101	-0.2	128.9	2.1023
0.5	2.0730	2.0707	-0.1	118.3	2.0623
1.0	1.9937	1.9917	-0.1	98.1	1.9856
1.5	1.9045	1.9128	+0.5	81.8	1.9078
2.0	1.8355	1.8339	-0.1	68.2	1.8300
2.5	1.7601	1.7550	-0.3	56.9	1.7522
3.0	1.6580	1.6760	-1.1	47.4	1.6744
3.5	1.5898	1.5971	+0.5	39.5	1.5966
4.0	1.5369	1.5182	-1.2	33.0	1.5188

that at the point of intersection  $\log \Phi_{35}^{\circ} = \log \Phi_{45}^{\circ} = \log \Phi_{50}^{\circ}$  and solving equation (10) for  $b$ . From the average value of  $-8.11$  the corresponding fluidity was calculated to be 2553 rhes ( $\log \Phi = 3.407$ ). From these values the slope of each curve can be calculated; thus for example, the slope of the curve at 35° is

$$\beta = \frac{\log \Phi - \alpha}{b'} = \frac{3.407 - 2.1496}{-8.11} = -0.155$$

For 45° and 60° the corresponding values are  $-0.145$  and  $-0.128_5$ . Assuming as a first approximation that the slope  $\beta$  is linear over a limited range of temperature, we have obtained

$$\beta = -0.1934 + 0.00108 t$$

TABLE VIII  
*Fluidity of Solutions of Isoelectric Gelatin*

After Loeb.

Concentration	Log $\Phi_{45}^\circ$ observed	Log $\Phi_{45}^\circ$ calcu- lated equation (10)	Deviation	$\Phi_{45}^\circ$ observed	Log $\Phi_{45}^\circ$ calculated equation (11)
<i>vol. per cent</i>			<i>per cent</i>		
0.0	(2.2227)	2.2290	+0.4	169.4	2.2227
0.25	2.1921	2.1927	0.0	155.9	2.1865
0.5	2.1545	2.1564	+0.1	143.3	2.1503
1.0	2.0877	2.0837	+0.2	121.2	2.0779
1.5	2.0092	2.0117	+0.1	102.7	2.0055
2.0	1.9431	1.9385	-0.2	86.6	1.9331
2.5	1.8715	1.8658	-0.3	73.4	1.8607
3.0	1.7818	1.7932	+0.7	62.1	1.7883
3.5	1.7176	1.7204	+0.2	52.5	1.7159
4.0	1.6567	1.6480	-0.5	44.5	1.6435

TABLE IX  
*Fluidity of Solutions of Isoelectric Gelatin*

After Loeb.

Concentration	Log $\Phi_{60}^\circ$ observed	Log $\Phi_{60}^\circ$ calcu- lated equation (10)	Deviation	$\Phi_{60}^\circ$ observed	Log $\Phi_{60}^\circ$ calculated equation (11)
<i>vol. per cent</i>			<i>per cent</i>		
0.0	(2.3290)	2.3547	+1.1	226.3	2.3290
0.25	2.3054	2.3216	+0.7	209.7	2.2969
0.5	2.2786	2.2884	+0.5	194.3	2.2648
1.0	2.2360	2.2221	-0.7	166.7	2.2005
1.5	2.1634	2.1558	-0.4	143.1	2.1362
2.0	2.0940	2.0895	-0.2	122.8	2.0720
2.5	2.0337	2.0232	-0.5	105.4	2.0078
3.0	[2.0196]	2.9569	-3.1	90.3	1.9435
3.5	1.8969	1.8906	-0.3	78.9	1.8792
4.0	1.8076	1.8243	+0.7	66.6	1.8150

where  $t$  is the temperature Centigrade. Equation (10) may now be written

$$\log \Phi = \log \Phi_1 - (0.1934 - 0.00108 t) b \quad (11).$$

Using these two constants and the known fluidities of water, the calculated values of the fluidity of the solutions given in the final column of the table were obtained.



We have presented evidence that the fluidity-concentration coefficient of the blood proteins  $-\partial\Phi/\partial b$  is a constant; Loeb's conclusion and our analysis

TABLE X  
*The Values of the Constants  $\alpha$  and  $\beta$  of Equation (10) for Isoelectric Gelatin*

Temperature	$\alpha$	$\beta$	$\alpha/\beta$
°C.			
25	2.0511	0.1679	12.2
35	2.1496	0.1579	13.6
45	2.2290	0.1453	15.4
60	2.3547	0.1326	17.8

If it is true that the fluidity curves of gelatin at different temperatures are to be conceived as meeting at zero fluidity, it is also interesting to note that assuming that the above logarithmic curve can be trusted, when  $\log \Phi = 0$  the fluidity of the solution must be unity. Thus the ratio  $\alpha/\beta$  given in Table X has interest, since it expresses the relation between the concentrations required for unit fluidity at the different temperatures. This relation may be of value in testing the validity of the formula.

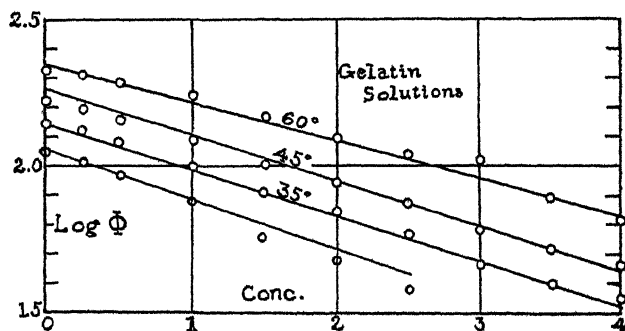


FIG. 2. Logarithmic fluidity (Briggsian) of solutions of isoelectric gelatin at various concentrations and temperatures (after Loeb).

of the data indicate that it is variable in the case of gelatin. If this coefficient varies directly as the fluidity, we have

$$-\partial\Phi/\partial b = \lambda\Phi \quad (12)$$

On integration of this equation

$$\ln \Phi = \ln \Phi_1 - \lambda b$$

whence

$$\Phi = \Phi_1 e^{-\lambda b} \quad (13)$$

or

$$\log \Phi/\Phi_1 = \frac{-\lambda b}{2.303} \quad (14)$$

so that the new fluidity-concentration constant  $\lambda$  can be obtained. It is instructive to note the great difference in the two types of protein, according to the above reasoning. Egg albumin lowers the fluidity to zero at 28.4 per cent, hence each per cent lowers the fluidity by  $\beta = 1/28.4 = 3.5$  per cent of the fluidity of the *solvent*, independent of the temperature. In other words, at low concentrations, egg albumin behaves rheologically like an inert finely divided suspension in the medium, since it merely decreases the *free volume* of the medium in proportion to the volume present, thereby obeying the Bachinskii law.

If in the non-hydrating type of protein,  $\beta = \Phi_1/b'$ , then the fluidity at any concentration  $b$  is

$$\Phi = \Phi_1 - \Phi_1/b' \cdot b \quad (15)$$

or

$$\frac{\Phi_1 - \Phi}{\Phi_1} = \frac{b'}{b} \quad (16)$$

On the other hand, gelatin is strongly lyophilic in that water actively swells it and at least partially is immobilized thereby, forming "hydrates." The number of molecules of water in these complexes is large in dilute solutions, but according to the exponential relation of equation (13) it becomes rapidly less as the concentration increases and the fluidity never reaches a zero value as it does with egg albumin. Thus a 1 per cent solution of gelatin lowers the fluidity of water 30 per cent but a 2 per cent solution lowers it not 60 but 51 per cent. These relations are shown graphically in Fig. 3. From equation (14), we calculate the value of  $\lambda$  to be  $-0.370$  at  $35^\circ$ ,  $-0.336$  at  $45^\circ$ , and  $-0.298$  at  $60^\circ$  corresponding to smaller hydration at the highest temperature. We have thus far no relation between  $\beta$  and  $\lambda$ .

#### *Serum Albumin (Crystallized)*

On page 90 of our Paper III we gave two closely agreeing values of  $\beta_{37^\circ}$  for horse serum albumin, yet at the same time we noted that Chick and Lubrznska (2) have given values for the viscosity of crystallized horse serum at  $25.4^\circ$  which are exceptional, the fluidity-concentration not being linear. By neglecting for the time being the three highest concentrations, we have, however, found that the remaining data fit the following formula:

$$\Phi = 112.7 - 5.208 b$$

The value of  $b'$  is 21.6 per cent and of  $\beta_{37^\circ}$  6.8 to be compared with the values from the data of Fahey and Green of 6.61 and 6.26. The observed and calculated values are given in Table XI and Fig. 4. The average percentage deviation of the first five solutions, including pure water, is 0.5; nevertheless the exclusion of the three highest concentrations is unjustifiable without sound

reason since the discarded data may be the most significant. This suggests at once an investigation to determine whether in this case, and others like it,

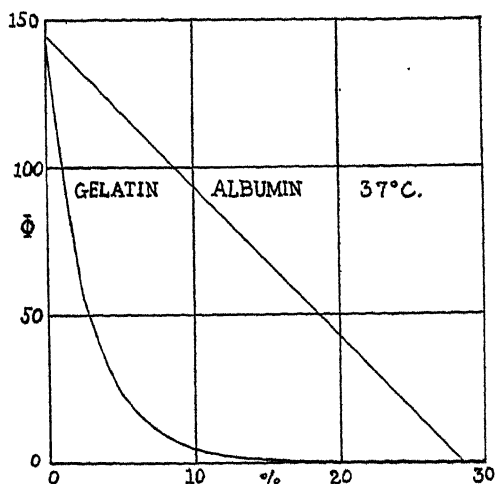


FIG. 3. Comparison of the types of curve exhibited by gelatin and albumin. Values calculated by the logarithmic and linear formulas respectively. It is also noted that the concentration of the albumin is in weight per cent. This does not affect the shape of the curve but it does affect the constant  $\beta_{37^\circ}$ .

TABLE XI

*The Fluidity of Solutions of Serum Albumin (Crystallized) at 25.4°C.*

After Chick and Lubrznska.

The calculated values are obtained by the use of the formula  $\Phi = \Phi_1 - \beta b$ .

Protein per cent by weight $b$	Relative viscosity $\eta/\eta_0$	Fluidity observed $\Phi_0$	Fluidity calculated $\Phi_c$	$\Phi_c - \Phi_0$
0.00	1.00	112.9	112.7	-0.2
2.59	1.13	99.4	99.2	-0.2
5.19	1.32	85.6	85.7	+0.1
10.45	1.95	57.9	58.3	+0.4
14.54	3.02	37.4	37.0	-0.4
17.85*	4.76	23.7	19.8	-3.9
19.24*	5.95	19.0	12.5	-6.5
20.65*	7.54	15.0	5.2	-9.8

$\beta = 5.21$ ,  $b' = 21.6$  per cent,  $\beta_{37^\circ} = 6.8$ .

\* These data were not used in calculating the constants of the equation. The residuals,  $\Phi_c - \Phi_0 = \Delta$  may be calculated by the equation  $\Delta = 30.8 - 1.95 b$ .

the fluidity is affected by the shearing stress used to bring about the shear. If it is, it will prove that a portion of the shearing stress is used up in some new way, as in overcoming internal static friction, which is a *yield stress* (8). These

problems connected with colloid solutions are accentuated when the concentration of colloid is high and the solubility of the colloid is low. We will now consider briefly two examples from the work of Chick (9) on pseudoglobulin and euglobulin.

*Pseudoglobulin and Euglobulin*

Albumin is soluble in water in all proportions and its fluidity is not greatly affected by salts like ammonium sulfate; pseudoglobulin can be dissolved in

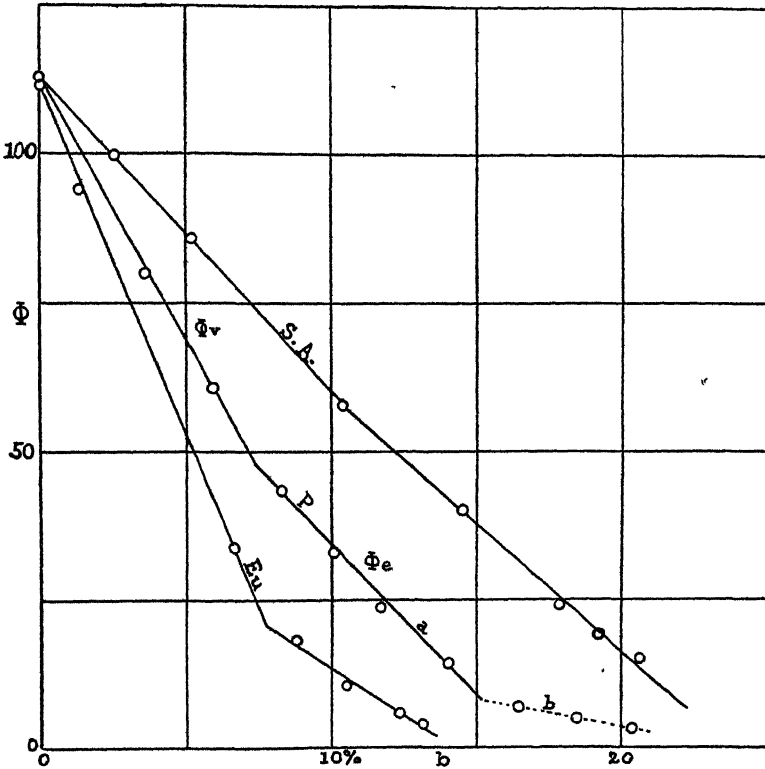


FIG. 4. Fluidity of solutions of serum albumin, pseudoglobulin, and euglobulin.

water but the fluidity of the solution is greatly affected by ammonium sulfate; however, the euglobulin was held in solution only by means of a 3.6 per cent solution of sodium chloride. The data for these last are given in Tables XII and XIII. In the case of serum albumin we observed that the linear fluidity-concentration formula could be applied up to a certain concentration after which it failed quite badly. The same observation may be made in regard to pseudoglobulin, except that the concentration at which failure begins is progressively lower, 17.85 in serum albumin, 10.04 in pseudoglobulin, and 8.86 per cent with euglobulin (Fig. 4). Since most of the observations were made at

higher concentrations, it may seem to the reader rather fruitless to attempt to apply the linear equation over so limited a range. If, however, there is a change of régime of flow from say viscous to plastic, a different formula may

TABLE XII

*The Fluidity of Solutions of Pseudoglobulin (Horse) at 25°C.*

After Chick (10).

Protein per cent by weight $b$	Relative viscosity $\eta/\eta_0$	Fluidity observed $\Phi_0$	Fluidity calculated $\Phi_c$	$\Phi_c - \Phi_0$
0.0	1.00	111.9	110.8	-1.1
3.61	1.40	79.9	81.0	+1.1
5.95	1.85	60.5	61.6	+1.1
8.32	2.59	43.2	42.0	-1.2
10.04	3.43	32.6	27.8	-4.8
11.82	4.70	23.8	13.1	-10.7
14.06*	8.05	13.9	-5.3	-19.2
16.43*	13.74	6.81	-25.0	-31.8
18.45*	23.31	4.80	-41.72	-46.5
20.37*	38.79	2.89	-57.60	-60.5

$\beta = 8.54$ ,  $b' = 13.0$  per cent,  $\beta_{37^\circ} = 11.1$ .

\* These data were not used in calculating the constants of the equation. The residuals.  $\Phi_c - \Phi_0 = \Delta$ , may be calculated by the equation  $\Delta = 76.9 - 6.72 b$ .

TABLE XIII

*The Fluidity of Solutions of Euglobulin in 3.6 Per Cent Sodium Chloride Solution at 25°  
pH  $10^{-5.8}$*

After Chick (9).

Protein per cent by weight $b$	Relative viscosity $\eta/\eta_0$	Fluidity observed $\Phi_0$	Fluidity calculated $\Phi_c$	$\Phi_c - \Phi_0$	
0.0	1.00	111.3	110.3	-1.0	Equation (20)
1.36	1.19	93.5	94.5	1.0	
6.58	3.32	33.5	33.5	0.0	
8.86*	6.23	17.9	16.6	-1.3	Equation (22)
10.57*	10.87	10.2	11.3	1.1	
12.32*	20.56	5.4	5.9	0.5	
13.20*	29.56	3.8	3.2	-0.6	

\* See text, page 621.

be needed for each régime and the linear formula may be very useful for predicting the fluidities within each range. If there is a change of régime above a certain critical concentration, it is important to recognize that fact clearly. Although the data may be too scanty to permit us to attain the certainty that we desire, we see no reason why additional data will not give proof for or against.

*Plastic Flow*

In our review of the older work on the rheology of the blood the effort has been made to avoid the discussion of plastic flow, but it is now necessary to introduce some of the elementary conceptions. Clerk Maxwell was the first to express clear ideas as to the nature of plastic flow in his Theory of heat in 1862. He said in part, "if the form of the body is found to be permanently

TABLE XIV

*The Fluidity of Solutions of Gelatin As Calculated from the Linear Fluidity-Concentration Equations (20), (21), and (22)*

Gelatin	Fluidity							
	25° obs.	25° calc.	35° obs.	35° calc.	45° obs.	45° calc.	60° obs.	60° calc.
<i>vol. per cent</i>								
0.0	111.9	111.8	138.4	139.4	167.0	167.1	213.3	213.0
0.25	102.7	102.8	130.1	129.1	155.7	155.6	202.0	202.3
0.50	93.3	93.7	118.3	118.8	142.7	144.1	189.9	191.7
1.0	76.0	75.6	98.6	98.1	122.4	121.0	172.2	170.4
								Equation 20
1.5	59.2	59.2	80.3	78.6	100.2	99.1	145.7	149.2
2.0	47.7	47.6	68.5	68.8	87.7	87.5	124.2	122.5
2.5	38.0	36.0	57.6	59.0	74.4	75.9	108.0	108.0
3.0	22.6	24.4	45.5	49.3	60.5	64.3	93.2	93.5
3.5	—	—	38.9	39.6	52.2	52.7	78.9	79.0
4.0	—	—	34.4	29.8	45.4	41.2	64.2	64.5
								Equation 22
	Percentage deviation							
Equation 20	0.3		0.8		0.3		0.8	
Equation 22	2.2		4.4		4.4		0.6	

altered when the stress exceeds a *certain stress*, the body is said to be soft or *plastic* and the state of the body when the alteration is just going to take place is called the limit of perfect elasticity." (The italics are ours.) Maxwell did not himself attempt to formulate his ideas of plastic flow but due to his suggestion the attempt was made by Butcher (10) in 1876. In 1890, Schwedoff (11) studied the phenomena of flow of colloids and published a paper under the title Experimental studies on the cohesion of liquids. This title and the omission of any word suggesting plastic flow in his papers tended to prevent early recognition of the fact that he first formulated the fundamental law of plastic flow and at the same time obtained its experimental verification. It is of course only natural that a physical property should be well established before it is christened, so also in the fundamental paper of Poiseuille establishing the

nature of viscous flow the word viscosity is not mentioned. The formulation of Schwedoff in our own nomenclature is

$$v = \mu(F - f)r \quad (17)$$

where  $v$  is the velocity in centimeters per second given to either of two parallel planes, separated from each other by the distance  $r$ , by a shearing stress of  $F$  dynes per cm.<sup>2</sup>,  $f$  being the yield stress and  $\mu$  the mobility. The mobility has the same dimensions as the fluidity but the name seems necessary to distinguish it from the commonly used quantity which should be always referred to as "apparent fluidity," and is not a constant at all but varies with the shearing stress. The yield stress was first called by Bingham the internal friction and later the yield value.

When solid spheres of equal radii are suspended in a viscous liquid, the fluidity is decreased and reaches a zero value when the percentage of liquid may have a very considerable value. In tetrahedral close packing the volume of liquid in the pore spaces is  $1 - \frac{\pi}{3\sqrt{2}}$  which is equivalent to 26 per cent of the unit volume, but in that form of close packing the shear would not be possible without dilation or the shearing of the particles themselves. That interlocking would cease with cubical close packing when the pore space would be  $1 - \frac{\pi}{6}$  or 47.6 per cent, at least enough to permit shearing, but a yield value might occur locally when the pore space is much greater than 50 per cent.

Emulsions differ rheologically from suspensions in that shearing is not out of the question when the percentage of the disperse phase rises above 74 per cent by volume because the drops of liquid are capable of being sheared and there would be an indication of a change of régime as the concentration reached the concentration where the spherical drops would just touch each other and fill the entire space; *i.e.*, 52 per cent by volume. Equation (15) for suspensions shows that the lowering of the fluidity by solid particles is quite what one might expect from the law of Bachinskii

$$\Phi = A(V - \Omega) \quad (18)$$

where  $\Omega$  is the limiting volume,  $V$  the observed molecular volume, and  $V - \Omega$  is known as the *free volume*. In suspensions it may also be said that the fluidity is proportional to the free volume, if we assume that the liquid in the pore spaces at close packing is all *bound*. For the ideal suspension of equal spheres, there appear the following simple laws. (1) The fluidity of the suspension will be independent of the shearing stress in dilute suspensions only. (2) The fluidity will be independent of the radius of the particle within wide limits, but Brownian movement should be negligible and the particles must be small

compared with the capillary diameters of the viscometer. (3) If the particles of the ideal suspension were inert, *i.e.* without interaction upon each other, the fluidity concentration curves would be linear and the concentration of zero fluidity would be independent of the substance. (4) The fluidity of a suspension is therefore completely determined in the viscous régime by a knowledge of the fluidity of the continuous phase at the temperature of observation and of the concentration of zero fluidity. Perhaps it goes without saying that these laws do not apply to polar substances, to suspensions at different pH, to particles which are not spherical and, especially of interest to us now, not to emulsions. For emulsions it is necessary to take into account the fluidity of the disperse phase.

As a first approximation, we shall assume that the more viscous phase follows a law exactly similar to the more fluid phase, the fluidity of the former being

$$\Phi_v = \Phi_1 - \frac{\Phi_1}{b'} b \quad (19)$$

and that of the latter

$$\Phi_p = \Phi_2 - \frac{\Phi_2}{a''} a \quad (20)$$

Since fluidities are additive, the fluidity of the mixture  $\Phi_e$  is

$$\begin{aligned} \Phi_e &= \Phi_v + \Phi_p \\ &= \Phi_1 + \Phi_2 - \frac{\Phi_2}{a''} - \left( \frac{\Phi_1}{b'} - \frac{\Phi_2}{a''} \right) b \end{aligned} \quad (21)$$

where  $a'' = 1 - b''$ , the concentration of the more viscous phase at which the fluidity is zero. Fluidity cannot have a negative value and therefore the above equation represents three straight lines.

Returning to the discussion of pseudoglobulin and euglobulin solutions, we point out that Chick (9) p. 270 *ff.* made much of the two-phase relation. Hatschek (12) had already observed the rapid increase in viscosity which occurs in an emulsion of oil in water when the disperse phase occupies more than half of the volume. Chick noted that as the concentration of protein was increased, the volume occupied by 1 gm. of protein at first increased and then became constant, which coincides closely with what we are describing as a change in régime of flow. Those observations yielding constant specific volumes, we have starred. Chick gives the volume of water associated with 1 gm. of protein at 25° to be, as follows: serum albumin 2.09, pseudoglobulin 3.78, euglobulin 5.81, and sodium caseinogenate 8.63. Since this water thus associated with the proteins is bound and therefore partially immobilized, these values should be closely related to our fluidity-concentration coefficients.



*Gelatin Solution As a Two-Phase System*

Gelatin is the only protein to which we have been able to apply the logarithmic formula of Arrhenius but even in that case the log curves cannot be extrapolated from even so dilute a solution as 0.25 per cent (Fig. 2); at 60° the 0.25 and 0.50 per cent solutions seem to fall not on a straight line but on a smooth curve to include the pure solvent. A more serious matter may be the lack of linearity in the log curve at 25° above the 1 per cent solution. The logarithmic formula is convenient to use, but the question arises as to whether it is reliable and can be used beyond the observed range, as in Fig. 3, and still correspond to reality. The logarithmic formula certainly does not suggest any change of régime when the swollen particles are close packed. We are therefore justified

TABLE XV  
*Constants for Equations (20), (21), and (22) for Gelatin*

Temperature	Equation (20)			Equation (22)			Equation (21)			$\beta\pi^\circ$
	$\alpha_v$	$\beta_v$	$b'_v$	$\alpha_e$	$\beta_e$	$b'_e$	$\alpha_p$	$\beta_p$	$b'_p$	
°C.										
25	111.8	36.3	3.08	94.0	23.2	4.05	-17.8	-13.1	1.36	46.8
35	139.4	41.3	3.37	107.8	19.5	5.53	-31.6	-21.8	1.45	42.7
45	167.1	46.1	3.63	133.8	23.2	5.78	-33.1	-22.8	1.45	39.7
60	213.0	42.5	5.00	180.5	29.0	6.21	-32.5	-13.5	2.40	28.8
Serum albumin	113.0	5.29	21.3	100.2	4.21	23.8				6.8
Pseudoglobulin	111.3	8.54	13.0	(a) 84.4	5.07	16.6				11.1
				(b) 23.2	1.00	23.2				
Euglobulin	110.3	11.7	9.4	44.0	3.09	14.2				15.3
Sodium caseinogenate	104.5	16.4	6.4	41.3	3.89	13.6				22.6

in trying formula (21) to preserve the linear fluidity-concentration relationship which seems to be so general at low concentrations. See Table XIV.

We find that the fluidity-concentration curves of gelatin are all linear within experimental error up to 1.5 per cent, and this is nearly one-half of the available range. The constants for the equation

$$\Phi_v = \Phi_1 - \frac{\Phi_1}{b'} b \quad (22)$$

$\Phi_1$  and  $\frac{\Phi_1}{b'}$  are given in Table XV for each temperature. The fluidities calculated for concentrations higher than 1.5 per cent are much too low, the above formula applying to an emulsion in which the particles of the disperse phase are not appreciably deformed. When the drops are crowded together their

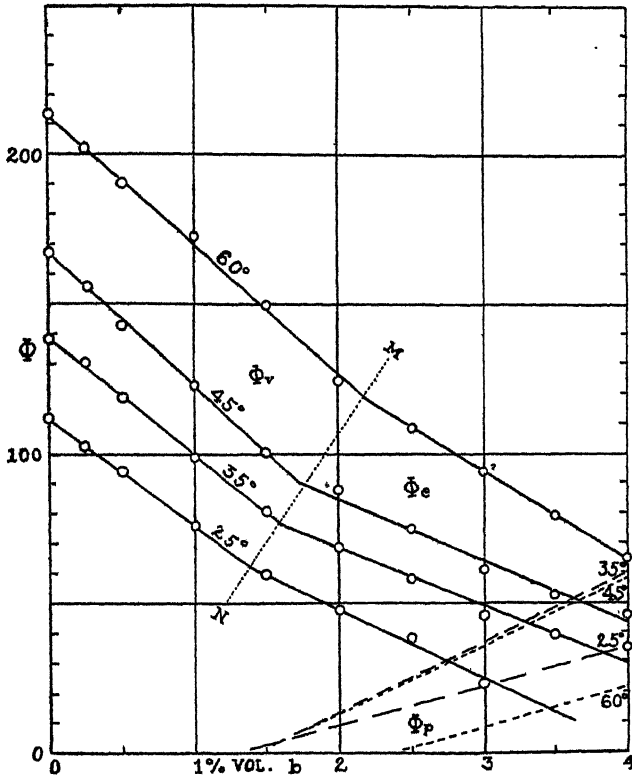


FIG. 5. Fluidity of solutions of isoelectric gelatin at various concentrations and temperatures, showing the use of differentiation of the viscous and plastic régimes with the use of formulas (20), (21), and (22). The disperse phase contributes  $\Phi_p$  to the fluidity in the viscous régime  $\Phi_v$ , giving the fluidity in the plastic régime to be  $\Phi_e$ .

deformation contributes a certain amount to the fluidity  $\Phi_p$ . If the fluidity of the emulsion  $\Phi_e$  is the result of the summation of the two deformations, then  $\Phi_e = \Phi_v + \Phi_p$  and

$$\Phi_p = \Phi_2 + \frac{\Phi_2}{a''} a \quad (23)$$

where  $a$  is the fraction of the continuous phase and  $a''$  is the particular value of  $a = 1 - b$  where the fluidity is zero; the  $\Phi_p$  curves are indicated by dashes in Fig. 5. The  $\Phi_e$  curve is the best one to fit the fluidity data *above* the change of régime, the equation being

$$\Phi_e = \Phi_1 + \Phi_2 - \frac{\Phi_2}{a''} - \left( \frac{\Phi_1}{b'} - \frac{\Phi_2}{a''} \right) b \quad (24)$$

This study suggests that a yield value may appear in gelatin solutions at about 1.5 per cent concentration at 25°, 35°, and 45° but at 60° perhaps over 2 per cent. This can be easily verified, if true, preferably by using two or more shearing stresses in determining each point. In passing, it is suggested that the contribution of the disperse phase to the fluidity may find an important application in blood where the erythrocytes are numerous, readily deformable, and large in comparison with the diameter of the capillaries.

The above method of handling the data for gelatin is seen to possess several advantages in addition. The  $\Phi_0$  curves extrapolate to include pure water. The data at 25° appear to be quite regular and in line with the data at other temperatures. The slopes of the  $\Phi_0$  and  $\Phi_s$  curves are most nearly equal at 60° indicating that the gelatin solution is approaching the condition of a truly viscous liquid.

TABLE XVI

*The Fluidity of Sodium Caseinogenate Solutions 9.39 Per Cent at 25°C.*

After Chick and Martin.

Protein per cent by weight	Relative viscosity	Fluidity observed	Fluidity calculated	Deviation	
0.0	1.0	111.9	104.5	-7.4	Equation (20)
2.17	1.82	61.5	68.9	+7.4	
4.35	3.37	33.2	33.2	0.0	
6.05	6.12	18.3	17.7	-0.6	Equation (22)
7.06	8.48	13.2	13.7	+0.5	
8.49	13.66	8.19	8.21	0.0	
9.39	23.72	4.72	4.71	0.0	

Finally, sodium caseinogenate has been included in this study using the data of Chick and Martin (13) given in Table XVI, Fig. 6. Again we find the higher concentrations to lie on a straight line,  $\Phi = 41.3 - 3.89 b$ , the average error being less than 2 per cent. There are only two observations and pure water to determine the  $\Phi_0$  curve and they are not enough.

#### CONCLUSIONS

A study has been made of those proteins which might offer exceptions to the law that the fluidity of a protein solution is a linear function of the volume concentration; *viz.*, egg albumin, serum albumin, pseudoglobulin, euglobulin, gelatin, and sodium caseinogenate.

Solutions of egg albumin below 20 per cent by weight obey the above law but somewhat below 30 per cent the fluidities begin to be too high, presumably due to the contribution to the fluidity made by the deformation of the particles as they come into contact, as the fluidity approaches zero.

The fluidity of serum albumin solutions shows a similar behavior, being exceptional above 15 per cent in weight. Pseudoglobulin and euglobulin give fluidity-concentration curves (Fig. 4) which are linear up to about 2.5 per cent each in a total range of 20 and 14 per cent respectively. From this singular point both compounds show a second range which is linear. Pseudoglobulin is the only substance whose solutions seem to show a third linear range. We have also used the data of Chick and Martin for sodium caseinogenate and found evidence for two linear régimes.

It is desirable at this time to call attention to the measurements of the flow of glycogen solutions by Botazzi and d'Errico (14) which in Fluidity and

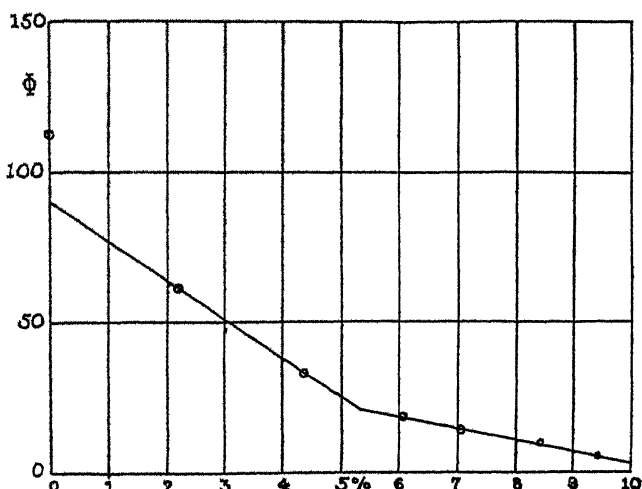


FIG. 6. The fluidity of solutions of sodium caseinogenate at 25°. The fluidity of pure water is indicated by a small circle but the extrapolation of the curve to 0.0 per cent suggests a  $N/2$  solution of sodium hydroxide.

plasticity, page 207, are expressed in rhes. The data show two linear fluidity curves of different slopes. In this case it was definitely known that the data for each curve were measured with different viscometers which suggested the possibility of an error in viscometry entering in to confuse the issue. We have no suspicions as to the reliability of the data studied in this paper; we only wish to caution the readers that our hypotheses based on these data must be regarded with due reserve until confirmed.

We have found a formula (11) based on the supposed linear relation between logarithmic fluidities and concentration which is convenient to use within the range, but close examination reveals that it does not reproduce the data for the higher concentrations at 25° nor does it permit extrapolation to pure water. It is not realistic enough because it does not contemplate any change of régime

in going from viscous to non-Newtonian or plastic flow. The formula does not apply to any other of the proteins studied in this paper nor to the great majority of proteins already reported as following the linear law. These are serious objections. We have therefore offered as an alternative a simple formula (24) according to which the fluidities are additive in the viscous régime. When the emulsoid particles approach close packing, they are deformed and this deformation contributes to the flow and the fluidity volume concentration curve is again linear. In fact, there may be one or more additional changes of régime.

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It is here noted that in calculating the fluidities given on page 86 of our Paper III, the data of Loeb, which should have been headed  $\log \eta/\eta_0$ , we employed as if headed  $\eta/\eta_0 - 1$ .
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